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MINERAL DEFICIENCY SYMPTOMS OF THE OIL PALM

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INTRODUCTION

Many oil palm plantations in the Belgian Congo have been established on poor soils, particularly those in the regions of the Kwilu and Kasai. The production of these plantations is generally low and the palms frequently exhibit foliar abnormalities, which are higly suggestive of deficiency diseases.

A considerable amount of work on foliar analysis is in progress and it is now possible to detect some of the mineral deficiencies in palms by this means. However, this method is expensive and there are, moreover, practical restrictions on the number of analyses that can be done. It is, therefore, held that the diagnosis of deficiencies by means of the visible symptoms is still of considerable importance.

Comparatively little work has been published on the deficiency symptoms of the oil palm. Bull [2] has recently described "Orange Frond Disease" which he has shown to be due to magnesium deficiency, and also "Confluent Orange Spotting" which he considers to be caused by potassium deficiency. Thompson [10] has reported that young palms which were grown in a nutrient solution without magnesium developed a chlorosis and browning whilst those grown on a nutrient solution without potassium developed a yellowing and the tips of the leaflets became scorched. A brief summary of the known deficiency symptoms of the oil palm has been given by Ferwerda [3].

During the last three years, information has been obtained, from both experiments and field observations, on the effect of mineral deficiencies on the oil palms. This information is included in the present paper.

METHODS

Most of the evidence relating to deficiency symptoms was obtained from two experiments, namely, a sand culture experiment and a "missing element" fertilizer trial.

1) *Sand culture experiment*

The design of the experiment was 6 × 6 latin square, which included the following treatments:

A. Palms watered daily with a complete nutrient solution.
B. Palms watered daily with a nutrient solution lacking nitrogen.
C. Palms watered daily with a nutrient solution lacking phosphate.
D. Palms watered daily with a nutrient solution lacking potassium.
E. Palms watered daily with a nutrient solution lacking calcium.
F. Palms watered daily with a nutrient solution lacking magnesium.

A single palm was planted in each of thirty-six bitumen, half two-hundred-litre drums filled with river washed quartz sand. The bottoms of the half-drums were holed to provide adequate drainage. The palms at planting were approximately nine months old (*i.e.* from germination of the seed), and treatments were not applied until growth had been resumed following transplanting. For the first sixteen months the palms were maintained in glass-roofed sheds and were then moved into the open as their size was such that it was impracticable to keep them any longer under cover.

Both the sand and the water used for making up the nutrient solutions were analysed for freedom from the experimental ingredients. Phosphate was present in the sand at a concentration of 80 ppm but potassium (< 50 ppm), magnesium (< 20 ppm), calcium (< 10 ppm) and nitrogen (< 5 ppm) could not be detected in either the sand or the water.

Treatments began on the 8 February 1954 and the solutions used were based on Hewitts' formula [6] with the following composition:

NO_3	15	milli-equivalents per litre
PO_4	4	,, ,, ,,
SO_4	3	,, ,, ,,
Ca	10	,, ,, ,,
Mg	3	,, ,, ,,
K	5	,, ,, ,,
Na	1.33	,, ,, ,,
H	2.67	,, ,, ,,
Fe	0.33	,, ,, ,,
Mn	0.02	,, ,, ,,
Cu	0.002	,, ,, ,,
Zn	0.002	,, ,, ,,
B	0.033	milli-mols ,, ,,
Mo	0.0002	milli-mols ,, ,,

A¹
A²
B¹
B²
Healty
S-deficient
Mg-deficient
C¹
C²
D¹
D²
D³
N-deficient
K-deficient
leaflets

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Each palm received 2 litres of the appropriate nutrient solution per day.

Leaflet samples were taken from the youngest open leaf at monthly intervals and analysed to determine the K, P, Mg and Ca content. The preliminary results of these analyses have been published by Broeshart [1] and further results will be published elsewhere.

2) *Missing element fertilizer trial*

This experiment was established at Brabanta in the Southern Congo to determine the cause of Little Leaf Disease which had been induced in a previous fertilizer experiment. Fourteen treatments were involved with four replications of each treatment and each plot contained sixteen palms. The plots were randomized. The treatments were:

1) Control – no fertilizer application.
2) Fertilizer application including N, P, K, Mg, Ca, Cu, Mn, B, Zn, Ni, Co and Mo.
3) as 2) without Mo.
4) ,, 2) ,, Co.
5) ,, 2) ,, Ni.
6) ,, 2) ,, Zn.
7) ,, 2) ,, B.
8) ,, 2) ,, Mn.
9) ,, 2) ,, Cu.
10) ,, 2) ,, Ca.
11) ,, 2) ,, Mg.
12) ,, 2) ,, K.
13) ,, 2) ,, P.
14) ,, 2) ,, N.

The rates of the fertilizer application, per (palm/annum) were:

	1953	1954
N.	1.5 kg sulphate of ammonia or 'Nitrochalk'	Same as 1953
P.	0.5 kg double superphosphate	,, ,, ,,
K.	0.75 kg sulphate of potash	,, ,, ,,
Mg.	1.0 kg sulphate of magnesium	,, ,, ,,
Ca.	3.0 kg ground limestone	Nil
Cu.	0.167 kg copper sulphate.	0.25% spray
Mn.	0.167 kg manganese sulphate	0.25% ,,
B.	0.017 kg zinc sulphate	1.00% ,,
Ni.	0.007 kg nickel sulphate	1.00% ,,
Co.	0.007 kg cobalt sulphate	0.10% ,,
Mo.	0.007 kg ammonium molybdate	0.10% ,,

The experiment was started during February 1953. The palms were then nearly five years old.

LEAF SYMPTOMS OF MINERAL DEFICIENCIES

1) *Nitrogen deficiency*

Abnormalities of the nitrogen-deficient palms of the sand culture experiment were first observed three months after the commencement of the treatments. All the leaflets of the youngest open leaf were a homogeneous yellow-green colour and the emergence of new leaves was greatly retarded. The older leaves became a pale green and in the final stages of the deficiency all the leaves were a conspicuous lemon-yellow colour. Desiccation of the leaflets was not usually associated with this condition. The main nerves of the leaflets were practically the same colour as the laminae and were commonly brittle. The leaf rachids were yellow to yellow green.

When the palms were moved to the open and not protected from the rain there was an improvement in their colour, the leaflets changing from yellow to pale green.

The palms which received no nitrogen application in the missing element experiment failed to develop any deficiency symptoms.

In the Belgian Congo, characteristic symptoms of nitrogen deficiency have not been observed in adult, plantation oil palms. Some young palms may show typical symptoms of nitrogen deficiency during the first two years following transplanting to the field. However, the yellow-green colour associated with this deficiency may be induced in some leaves by causes other than a lack of nitrogen in the soil, *i.e.* Vascular Wilt Disease and some forms of Bud Rot. Plate I illustrates leaflets from a nitrogen-deficient palm of the sand culture experiment.

2) *Phosphate deficiency.*

In the missing element experiment, the palms which did not receive phosphate exhibited a high incidence of premature desiccation of the older leaves.

No phosphate deficiency symptoms were observed in the palms of the sand culture experiment.

3) *Potassium deficiency*

Potassium deficiency symptoms first appeared in the sand culture palms after seven months. The earliest symptom was a

Plate I

Leaflets of oil palms showing different mineral deficiency symptoms.

chlorosis of the leaflets of the older leaves. This chlorosis appeared as a pale yellow colour when viewed by transmitted light. The yellowing started at the tips and progressed downwards along the margins of the leaflets. The main veins and parts of the laminae adjacent to them remained a normal, dark green colour. At a later stage of the disease, the tips and margins of the leaflets of the old leaves became desiccated but this was a gradual process as indicated by a very thin, pale brown "transition" zone between the desiccated and chlorosed tissue. After ten months, minute, longitudinal clear spots were observed scattered over the laminae of the leaflets of the youngest open leaves of the palms when viewed by transmitted light. This spotting persisted with the ageing of the leaf and all subsequent leaves were similarly affected.

In the later stages of the experiment, the potassium-deficient palms showed a marked shortening of the leaflets and rachids of the young leaves. As the number of the leaflets was normal, these leaves had a "bunchy" appearance. This symptom was very obvious in five of the six palms of the treatment but leaves produced later were less abnormal and recovery has been maintained to the present time.

Plate I illustrates leaflets from a potassium-deficient plam of the sand culture experiment. A "bunchy" type leaf is shown in Plate II.

The missing element trial confirmed the findings of the sand culture experiment in that the palms which did not receive potassium displayed the marginal chlorosis and desiccation of the leaflets of the old leaves. The presence of scattered, minute, clear spots on the leaflets of the young leaves was observed on some of the palms and there was a reduction in the length of the rachids without a reduction in the number of the leaflets.

The disease known as Confluent Orange Spotting and reported by Bull as being due to potassium deficiency, was not observed in either of the experiments.

4) *Magnesium deficiency*

The leaf symptoms associated with magnesium deficiency have been described by Bull [2] who cured affected palms by heavy applications of magnesium fertilizer.

The sand culture palms which received no magnesium developed typical symptoms after five months. Similar, striking symptoms

were also induced in the palms of the missing element trial which did not receive this element. The control palms of the latter experiment were similarly affected showing that the soils in the area of the experiment were naturally magnesium deficient.

An early symptom of magnesium deficiency is the appearance of an ochreous chlorosis in the laminae of the distal leaflets of the older leaves. This chlorosis is frequently most pronounced in the central part of the leaflets although this may be a secondary effect due to partial shading of the distal ends of the leaflets. When the deficiency becomes more accentuated the distal leaflets of the old leaves become yellow at the distal ends and this colour rapidly changes to a bright orange. The orange-coloured parts of the chlorosed leaflets appear to be of a homogeneous colour when seen against a bright background but close inspection shows that it is frequently mottled. With increasing severity of the disease, younger leaves become affected and the distal leaflets of the old leaves become a chocolate brown colour at their distal ends prior to desiccation. Desiccation is usually rapid and the distinctive chocolate brown transition zone between chlorosed and desiccated tissue is generally extensive. In the sand culture experiment the leaflet laminae of the outer whorl of leaves dried out and broke away leaving only the leaf rachids and the main nerves of the leaflets which also became desiccated at a later stage. The rachids of these leaves became more erect and their distal ends curled inwards until the palms assumed the symptoms of the extreme condition of the disease known as Plant Failure.

Plate I illustrates leaflets from a magnesium-deficient palm.

5) *Boron deficiency*

A note has been published by Ferwerda [4] in which it was stated that boron deficiency was the cause of Little Leaf Disease as described by Kovachich [9]. This was based on the results of the missing element experiment in which 53 of the 64 palms which did not receive boron developed Little Leaf symptoms. A more recent field experiment has induced 93% of the palms to develop Little Leaf Disease by applying all the essential elements except boron.

The first symptoms were apparent in the youngest open leaves: the distal leaflets were considerably shorter than normal and their

Plate II. Young leaf from potassium-deficient palm showing reduced growth of rachid and leaflets.

Plate III. Palm suffering from boron deficiency.

tips were commonly hooked. Other affected palms showed a pronounced reduction in the width of the distal leaflets. As the deficiency became more acute, there was a marked reduction in the length of the leaves and the development of the leaflets was abnormal, these being either corrugated, much reduced in size, or completely absent. The rachids of the badly affected leaves were callosed and stunted and the elongation of the young leaves was retarded. The abnormal leaves associated with boron deficiency differed from those of potassium deficiency in that the abnormalities were generally due to malformation rather than a simple reduction in growth. Rotting was limited to some leaflets of the younger spear leaves and was never extensive. No colour changes of the leaves were observed. Plate III shows a palm suffering from boron deficiency.

6) *Sulphur deficiency* *

Symptoms of this deficiency have been obtained in a factorial sand culture experiment and in a missing element trial. In the former experiment, twenty of the twenty-seven palms which did not receive sulphur showed symptoms after one year of planting as germinated seed and six months after treatment. In the latter the symptoms were almost general in the plots receiving complete fertilizer with omission of sulphur already four months after the establishment of the experiment.

The earliest sign of this deficiency was the general, pale green colour of the young leaves. This was followed by an interveinal chlorosis which was mottled or took the form of a yellow spotting, starting near the distal end of the leaflets and extending towards the base. The spots changed to an orange colour and red brown spots appeared in the centre. The latter spots enlarged and became a darker brown colour and neighbouring spots tended to coalesce to form narrow, irregular bands of necrosed tissue with chlorotic margins between the veins (Plate I). In the case of extreme deficiency the affected tissue became desiccated. The tissue immediately adjacent to the main nerve remained a darker green colour. Early symptoms of the deficiency could be seen on the youngest fully opened leaf and they became more severe with the ageing of the leaf. The median leaflets were most severely affected and symptoms have not been observed on basal leaflets up to the present time.

Similar symptoms to sulphur deficiency have been induced by excessive application of borax but the possible relationship between boron and sulphur in the nutrition of the oil palm still remains to be investigated.

7) *Other deficiencies*

No visual symptoms resulted from the omission of calcium in

* Experiments not described in this paper.

either the sand culture experiment or the missing element trial or from the absence of copper, manganese, zinc, nickel, cobalt, and molybdenum in the latter trial.

THE EFFECT OF MINERAL DEFICIENCIES ON GROWTH AND PRODUCTION

1) *Sand culture experiment*

The results obtained from the examination of dissected palms of each treatment are shown in Table I.

TABLE I

Results of the examination of three palms of each treatment of the sand culture experiment. (Figures represent the average per palm)

Treatment	A (complete)	B (—N)	C (—P)	D (—K)	E (—Ca)	F (—Mg)	Sig. diff. 0,05	Sig. diff. 0,01
Fresh weight of palm (g)	13190	2690++	14260	9770	13600	8640+	4180	5700
Fresh weight of roots (g)	3000	1010++	3140	1700++	2740	1520++	0920	1260
Fresh weight of inflorescences (g)	1122	0++	713	135++	769	23++	680	928
Number of main roots	156	90	115	72	112	90		
Number of leaves produced	34.6	26.6	36	41	35	40		
Length of 6th leaf (cm)	222	121	268	141	200	195		
Dry weight of palms (g)	4526	1079	4718	3226	4572	2441		
% age of total dry weight:								
a) Roots	23	48.2	22.5	17.5	22.5	19.7		
b) Stem	12.4	9.2	8.3	9.4	8.8	8.2		
c) Central spear	1.9	1.3	2.6	1.6	2.7	2.9		
d) Petioles	32.4	24.2	34.7	44.4	32.6	31.0		
e) Rachids	12.2	9.3	11.7	12.4	13.6	17.5		
f) Leaflets	12.9	7.7	14.2	13.2	14.4	20.9		
g) Inflorescences	8.0	0.0	5.8	1.4	5.5	0.5		

It will be seen that the weight of the palms of the —N and —Mg treatments are significantly low whilst the weight of the palms which did not receive potassium is also low but just fails to attain significance. An effect of not applying nitrogen, potassium and magnesium has been to depress root formation in all cases, both as regards the number of roots and the weight of the roots. The

nitrogen-deficient palms were found to have produced more roots in relation to their size than the palms of any other treatment when the dry weight of the roots was expressed as a percentage of the total dry weight of the palm.

The average number of leaves produced per palm of both the potassium- and the magnesium-deficient treatments tended to be higher than the other treatments whilst the leaf production of the nitrogen-deficient palms was very poor. Both the potassium- and the nitrogen-deficient palms produced shorter leaves than normal. The leaflets of the —Mg treatment palms accounted for a relatively high percentage of the total dry weight whilst the dry weight of the petioles of the —K treatment palms was also high for the weight of the palms.

The production of inflorescences was depressed in all the palms which did not receive the complete nutrient solution. The production of the palms which did not receive nitrogen, potassium and magnesium was significantly low.

2) *Missing element trial*

The length of the youngest open leaf of all the palms of this experiment was measured after 21 months. It was found that all the fertilizer treatments, except that where potassium was not included, caused a significant increase in the leaf length. The omission of potassium was significantly worse than complete fertilizing whilst the palms which did not receive phosphorus and boron had shorter leaves but the difference was not significant. The omission of calcium and nickel treatments were significantly better than the complete treatment under the conditions of the experiment.

During the first year of the experiment the omission of potassium and magnesium treatments both significantly depressed the production of male inflorescences compared with the full fertilizer treatment. In the second year the plots which did not receive phosphorus and boron showed a reduction in the production of male inflorescences. The omission of magnesium, boron and zinc resulted in a reduction in the number of female inflorescences, produced in the first year whilst the absence of potassium, magnesium and boron gave a significantly lower production of female bunches in the second year. Detailed production figures for this experiment have previously been published by Ferwerda [5].

3) *Other experiments*

Positive, significant response in production have been obtained, on various Congo soils, by the application of potassium, phosphate, magnesium and boron.

DISCUSSION

1) *'Confluent Orange Spotting'.*

Bull considered that this disease was caused by a deficiency of potash. As has been previously mentioned, this disease has not been induced by omitting potassium from the nutrient application. In the Belgian Congo, the disease is relatively rare and the distribution of the disease does not appear to be correlated with soil conditions. Furthermore, foliar analysis of leaflets from palms suffering from Confluent Orange Spotting and leaflets from neigbouring healthy palms have shown no differences in leaf composition and there was no indication that the palms were potash deficient.

The symptoms of the disease are considered to be more in keeping with a mycological disease and many fungi have been isolated from recently diseased tissue but inoculation experiments have, so far, failed to indicate a pathogen.

2) *'Plant Failure'*

This term was first used by Wardlaw [11] as a composite term to embrace a considerable range of symptoms thought to be of physiological origin but which had not been investigated. The term is now often used to indicate a state of extreme debility. Field experience has shown that several diseases may have these 'Plant Failure' symptoms, *i.e.* a tapering stem, the outer leaves desiccated and erect with the tips frequently incurled and chlorosed leaflets on the outer, non-desiccated leaves. It is known that a severe attack of the leaf disease Patch Yellow can cause the stem to taper and the outer leaves to become more erect. It is thought that the disease of the leaflets adversely effects the growth of the roots [7] and the poor rooting system is responsible for the reduced growth of the stem and the erect habit of the leaves. The sand culture experiment has demonstrated that Plant Failure can result from magnesium deficiency and it is interesting to note that the rooting of the palms was adversely affected by this deficiency. In plantations, it has been observed that many palms which have recovered

after two or more attacks of Little Leaf disease have later developed the typical aspect of Plant Failure palms. The chlorosis associated with this condition takes the form of a very pale, uniform, green colour, not unlike the symptoms of nitrogen deficiency. A symptom of Little Leaf disease is that the growth of the roots is arrested. There are indications that extreme potassium deficiency may result in a further type of Plant Failure. Thus, it would appear that the Plant Failure condition results from nutrient deficiencies which, among other things, restrict root development.

3) *'Little Leaf Disease'*

In a previous paper, Kovachich [8] reported that Little Leaf and Bud Rot were the same disease. This was based on the results of an extensive study in the field. The evidence of the missing element trial does not support this conclusion in that no extensive rotting of the central spear and bud was induced in the palms which did not receive boron. On the other hand, plantation palms suffering from the Little Leaf/Bud Rot complex have been cured by the application of borax. Thus, there can be little doubt that boron deficiency is chiefly responsible for this disease complex as found in the plantations in the Southern Congo but it is possible that a further deficiency plays a part in the rotting of the bud.

SUMMARY

Young oil palms in sand culture experiment developed symptoms of nitrogen, potassium and magnesium deficiencies involving a homogeneous yellowing, a marginal yellowing and an orange chlorosis respectively. All these deficiencies resulted in poor growth and a reduced root development. A further experiment showed that Little Leaf Disease was caused by boron deficiency. Magnesium and potassium deficiency symptoms were also induced in this experiment. Sulphur deficiency symptoms, consisting of a yellowing of the young leaves, an interveinal chlorosis and necrosis, were induced in a factorial sand culture experiment and a second missing element trial. Insufficient available nitrogen, potassium, magnesium and boron all have an adverse effect on the production of inflorescences.

It is considered that Plant Failure disease is caused by a poorly developed rooting system which may result from magnesium, boron and, possibly, potassium deficiencies.

No evidence has been obtained to support the view that Confluent Orange Spotting is caused by potassium deficiency.

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THE EFFECT OF LIMING A NEUTRAL SOIL ON THE UPTAKE OF MANGANESE BY PLANTS

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INTRODUCTION

Several workers [2 6 8 11] have observed temporary improvements in the appearance and yield of manganese-deficient cereals when heavy dressings of alkali are applied to the soil either as CaO or NaOH. This is an interesting effect, and it is perhaps surprising that there has never been a fundamental study of it. The outstanding problems seem to be whether the improvements after liming are associated with increased uptake of manganese by the plant, and if so, what is the mechanism.

The present study on the effect of liming consists of pot experiments. The main experiment was designed to compare the uptake of manganese by oats from increasing amounts of a synthetic δ-MnO_2 added to a soil at pH 7 and pH 8. In a parallel, but smaller experiment, the soil was first treated with sodium azide (following the success of sterilization reported elsewhere (see Ref. [10], pp. 238–239)). The soil selected for these experiments was collected from the field at Penola, in the south-east of South Australia, in January, 1953. The land there is a reclaimed swamp. The surface is uneven, being dotted with raised circular patches about fifteen yards in diameter, about a foot above the surrounding land and occupying about half the total area. The soil on these raised patches, which was used for this work, is a black clay of pH 7, of stable granular structure and containing 8 per cent organic matter; it overlies soft limestone at a depth of 9 inches, but itself contains only traces of $CaCO_3$. It is very well-drained in pots. The soil on the lower levels is similar on the surface, but is much deeper. Manganese deficiency disease of oats (Grey-speck) occurs especially on the

higher patches, the soil of which contains 3.5 parts per million of manganese extractable with ammonium acetate containing quinol [5].

METHODS

The pot experiments were started in 1953 and continued by resowing the same pots in 1954. The methods where different for the two years will be described separately.

Delta MnO_2, with an equivalent ratio of O : Mn of 1.99, was precipitated from a solution of $Ca(MnO_4)_2$ by adding a solution of $MnSO_4$ [1]. The term δ-MnO_2 is here used rather than manganous manganite of a former communication [4].

The culture pots were of glazed earthenware, and held 4.5 kg of soil. Before mixing, the soil was air dried and broken down to pass a 6-mm sieve. The δ-MnO_2 was intimately mixed throughout each 4.5-kg sample of soil to give pots containing 0, 10, 18, 32 and 60 parts of Mn per million of the soil. There were four pots of soil at each level of Mn, two of which were left at pH 7.0 and two of which were limed to pH 8.0 by mixing 17 g $Ca(OH)_2$ throughout the air-dried soil. Another series of cultures was similarly prepared with 0, 10, and 18 parts of Mn per million of the soil at both pH's 7.0 and 8.0, and 1 g of sodium azide in solution was mixed throughout each pot of soil. These cultures are referred to as 'sterilized'.

Superphosphate was mixed at the rate of 1.580 g throughout each pot of soil, which finally received a surface application of the following: KNO_3, 630 mg; $MgSO_4.7H_2O$, 157 mg; $ZnSO_4.7H_2O$, 14 mg; and $CuSO_4.5H_2O$, 14 mg. During May, 1954 this surface application was repeated together with one of 1.10 g of Na_2HPO_4 per pot. All reagents were free of Mn.

Water was added to the soil to make 30% by weight or 60% of the water-holding capacity, (*i.e.* total pore space), and fifteen seeds of "Algerian" oats, (a local variety highly prone to Grey-speck) were sown in each pot in mid-June. The number of seedlings was subsequently reduced to thirteen. The plants were grown in the open in a bird-proof enclosure in the grounds of the Agriculture School, Melbourne University, Victoria, but were protected from rain by a rolling tarpaulin. Pots were weighed at two-day intervals and watered by hand in an attempt to keep the soil at 60% water-holding capacity.

The plants were harvested at maturity in mid-December. Grain and straw were separated, dried at 105°C for 15 hours and weighed. Samples of the grain and straw were digested with a mixture of perchloric, nitric and sulphuric acids, and manganese was then estimated by the colorimetric—periodate method using a Hilger "Spekker" Absorptiometer.

RESULTS

Appearance of plants

During 1953, manganese deficiency symptoms were overcome at both pH 8 and pH 7 with 10 ppm Mn added to the soil. In the

absence of added MnO_2 the deficiency symptoms were less severe on plants grown at pH 8 than at pH 7. During 1954 the deficiency symptoms of plants grown at pH 8 were again less severe than at pH 7. However, in that year, symptoms appeared with 10 ppm of added Mn as well as where no Mn was added. At pH 8 the deficiency symptoms were overcome with 18 ppm of added Mn but at pH 7 32 ppm was needed.

The smaller experiment with sterilized soil was, in 1953, only partly successful, since the oats themselves were initially inhibited. However, the young plants did not die and after an hiatus of eight to ten weeks they made fair growth and finally flowered and produced grain. In 1954 the plants were not inhibited at all. During both 1953 and 1954 there were no symptoms of manganese deficiency even in the absence of added Mn.

Yields and uptake of Mn, 1953

The results of the main experiment of 1953 are shown in Table I and Figs. 1 to 5 inclusive. The yields with no Mn added were about

TABLE I

Year	MnO_2 added to soil, ppm Mn	Yield of oats on Penola soil (g dry matter)			
		Soil pH 7		Soil pH 8	
		Straw	Grain	Straw	Grain
1953	0	7.0$_5$	1.3	14.0	3.1
	10	22.0	8.8	21.7$_5$	12.7
	18	20.0	9.0	24.3	8.1
	32	18.5	10.0	23.3	12.0$_5$
	60	23.7	10.0	22.2	12.0
1954	0	5.0	nil	8.0	nil
	10	15.7	4.9	19.3	5.5$_5$
	18	17.7	8.1	20.6	5.8
	32	18.9	9.3	19.6	7.4
	60	21.2	9.3	20.8	9.7

twice as high at pH 8 as at pH 7. With 10 ppm Mn added to the soil, yields were much increased, and additional Mn up to 60 ppm caused no further increase; at all levels of added Mn there was no consistent difference between the yields at the two levels of pH.

However, the concentration of Mn in the plant, both in straw and grain, was throughout greater at the higher pH (Figs. 1 and 2). These concentrations increase with increasing Mn in the soil, up to

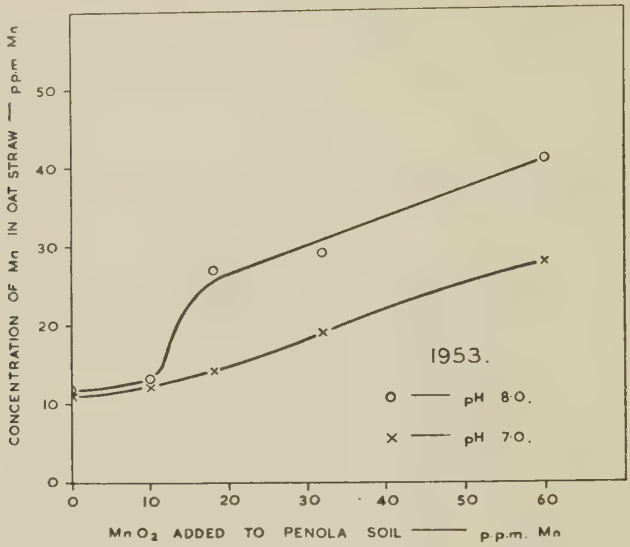

Fig. 1. Relationship between added MnO_2 and concentration of manganese in oat straw.

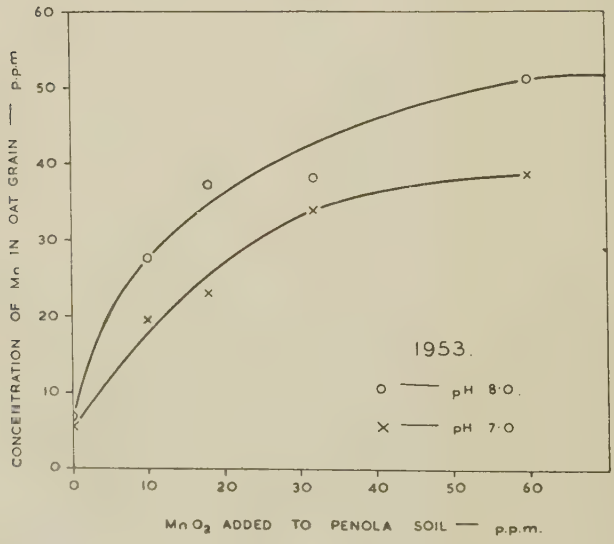

Fig. 2. Relationship between added MnO_2 and concentration of manganese in oat grain.

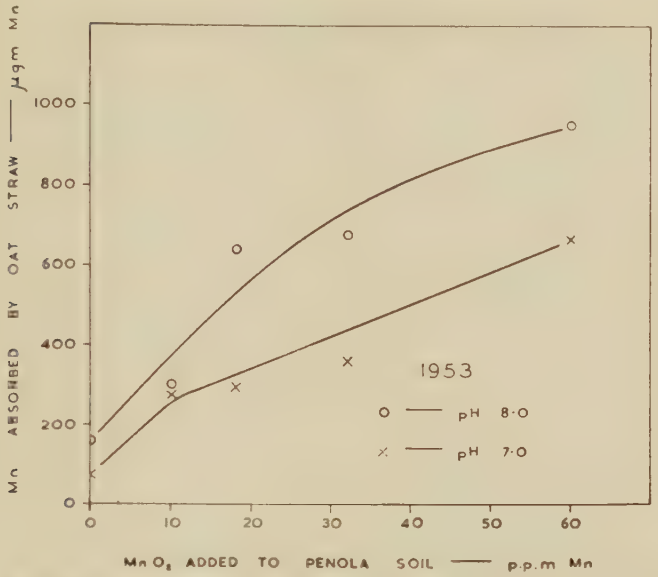

Fig. 3. Relationship between added MnO_2 and manganese absorbed by oat straw.

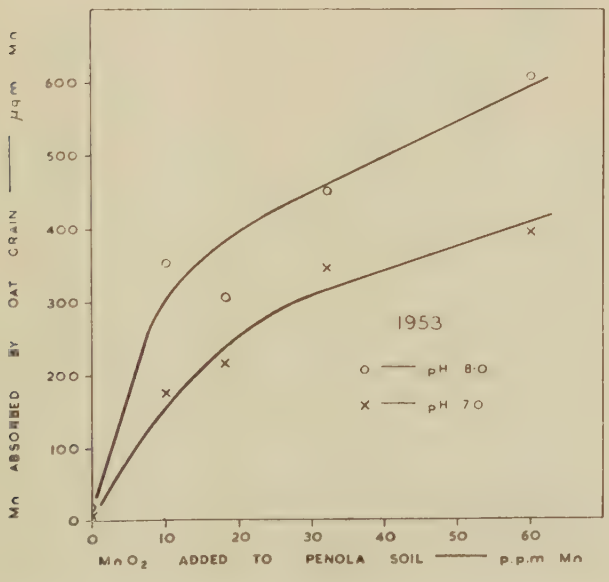

Fig. 4. Relationship between added MnO_2 and manganese absorbed by oat grain.

60 ppm of added Mn, at which level the figures are 41 ppm in the straw and 51 ppm in the grain at pH 8, and 28 and 39 ppm respectively at pH 7.

The total amount of Mn absorbed by the plants per pot is plotted

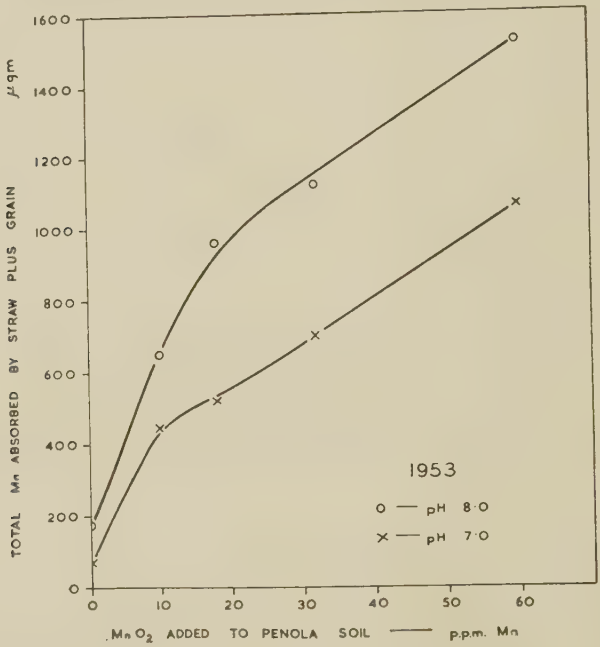

Fig. 5. Relationship between added MnO_2 and manganese absorbed by oats.

in Figs. 3, 4, and 5, which show how both native and added Mn were more available to the plant at the higher pH.

Yields and uptake of Mn, 1954

The results of the main experiment of 1954 are shown in Table I and Figs. 6 to 10 inclusive. The yields with no Mn added were again much greater at the higher pH, though both were low, and no grain at all was produced. With 10 ppm Mn added to the soil, yields were much increased, and additional Mn up to 60 ppm caused little further increase in total yield, except that the total yield with 10 ppm at pH 7 was noticeably lower than with more Mn added. Some of the yields of grain at pH 8 were rather low, in spite of the freedom from Grey-speck disease; an unknown disease had caused a number of empty heads on two of these pots.

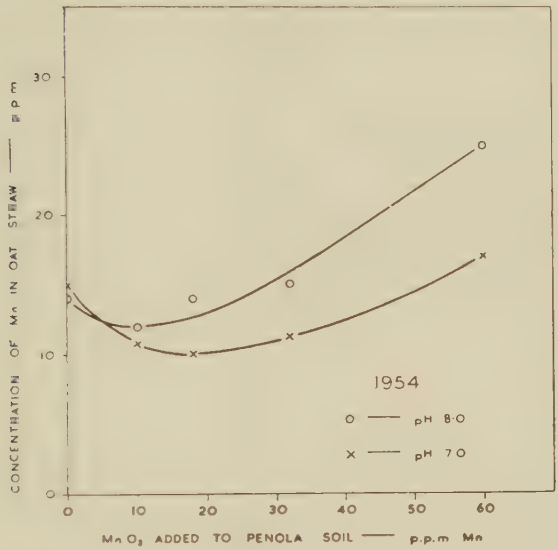

Fig. 6. Relationship between added MnO_2 and concentration of manganese in oat straw.

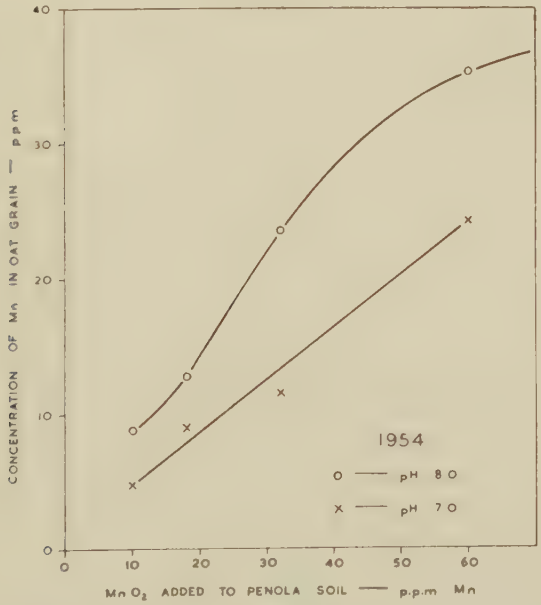

Fig. 7. Relationship between added MnO_2 and concentration of manganese in oat grain.

However, the main interest is in the concentration of Mn in the plant, which again was generally greater at the higher pH (Figs. 6 and 7). The one exception to this statement was the straw when no Mn had been added to the soil. As the curves of Fig. 7 show, this exception was accompanied by a remarkable *decrease* in the concentration of Mn in the plant as the deficiency was being overcome with added Mn; the total uptake of Mn (Fig. 8) shows the expected progressive increase with the addition of Mn to the soil. This minimum in Fig. 7 is characteristic of intense deficiencies [12].

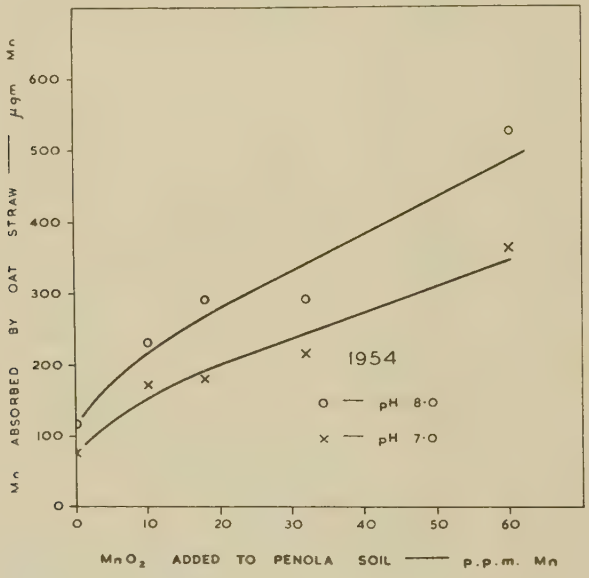

Fig. 8. Relationship between added MnO_2 and manganese absorbed by oat straw.

The total amount of Mn absorbed by the plants per pot is plotted in Figs. 8, 9, and 10, which show how both native and added Mn were again more available to the plant at the higher pH. All results point to a lower availability of both native and added Mn in 1954 than in 1953.

Yields and uptake of Mn from sterilized soil

The results of the smaller experiments of 1953 and 1954 are shown in Table II, and Figs. 11 to 14 inclusive. Although yields

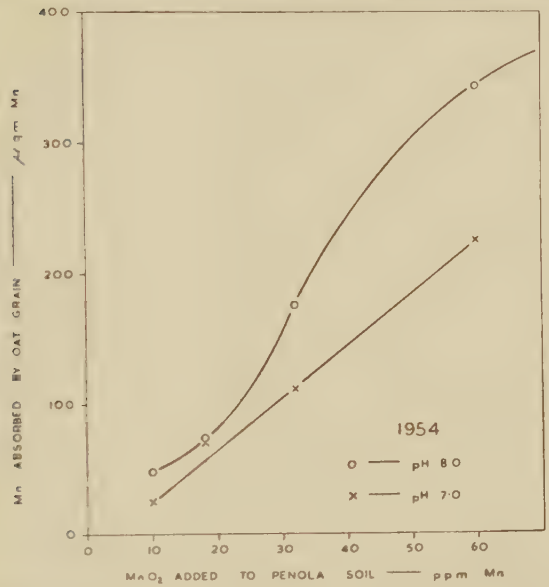

Fig. 9. Relationship between added MnO_2 and manganese absorbed by oat grain.

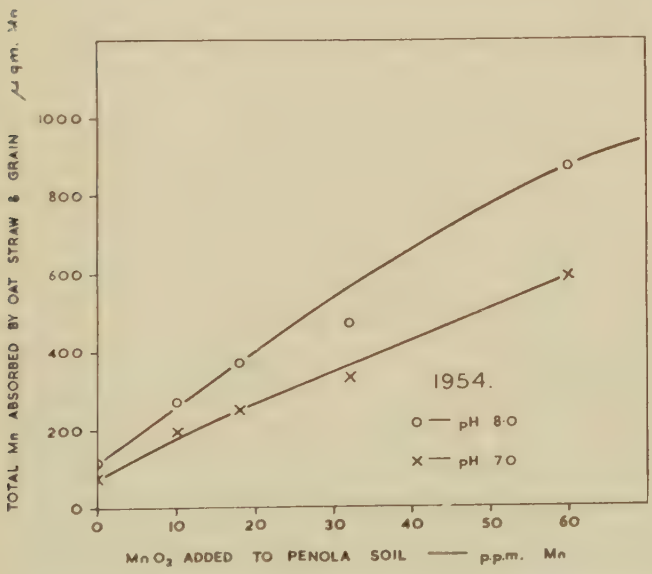

Fig. 10. Relationship between added MnO_2 and manganese absorbed by oats.

TABLE II

Yield of oats on sterilized Penola soil (g dry matter)					
Year	MnO_2 added to soil, ppm Mn	Soil pH 7		Soil pH 8	
		Straw	Grain	Straw	Grain
1953	0	7.6		7.3	
	10	7.9		7.7	
	18	8.4		7.5	
1954	0	26.8	7.8	24.6	10.0
	10	25.5	9.7	23.6	11.0
	18	24.7	9.3	25.9	9.2

were depressed in the year in which the sodium azide was added, they were approximately constant throughout. A particularly interesting effect emerged in the results of plant analyses for Mn. While, as usual, the concentration of Mn in the plants increased with increasing Mn in the soil, the relation to pH was reversed and there were greater concentrations of Mn in plants grown at pH 7 than at pH 8 (Fig. 11).

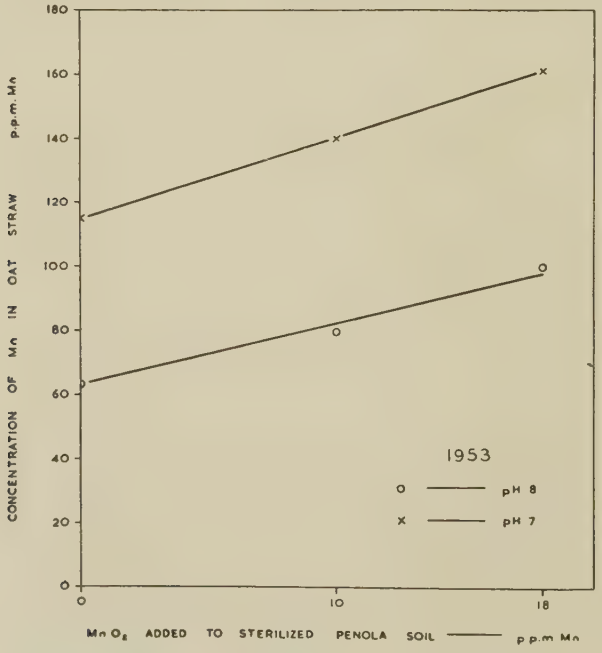

Fig. 11. Relationship between added MnO_2 in sterilized soil and concentration of manganese in oat straw.

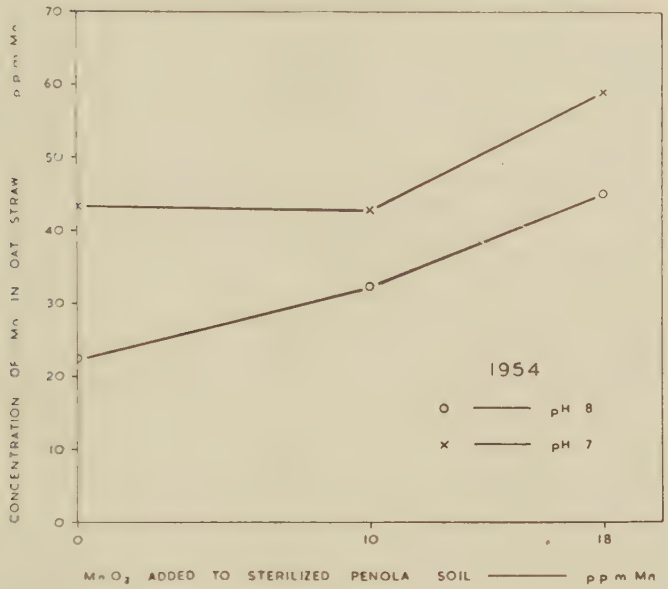

Fig. 12. Relationship between added MnO_2 in sterilized soil and concentration of manganese in oat straw.

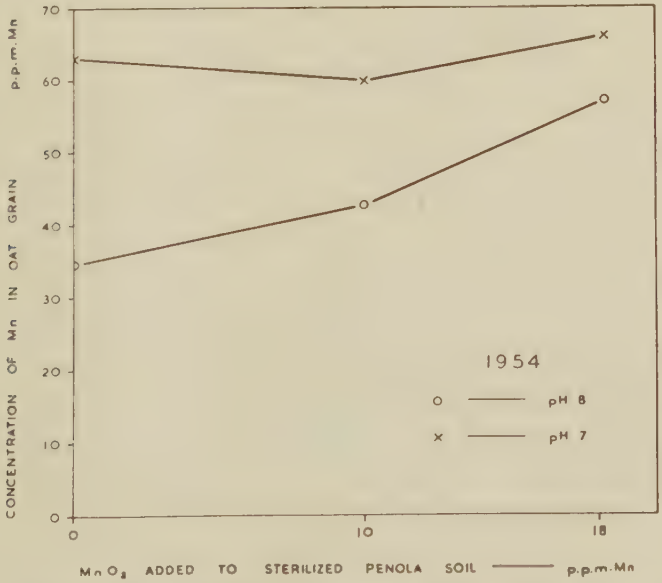

Fig. 13. Relationship between added MnO_2 in sterilized soil and concentration of manganese in oat grain.

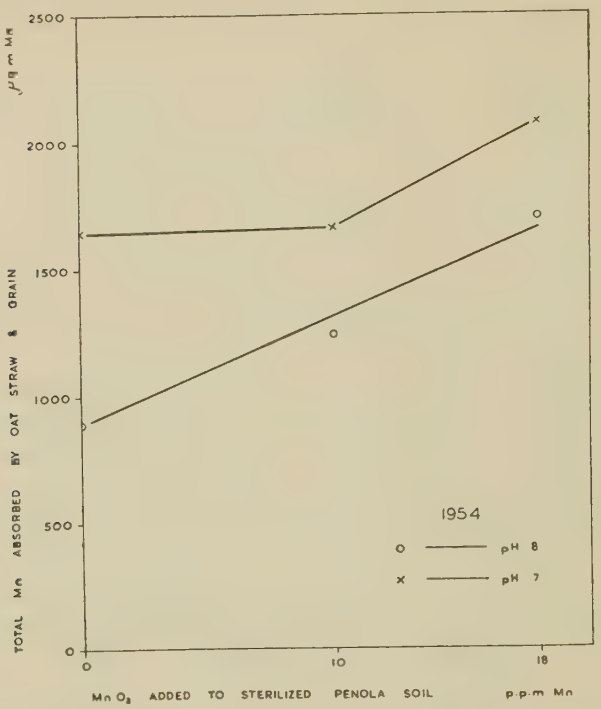

Fig. 14. Relationship between added MnO_2 in sterilized soil and manganese absorbed by oat plants.

The sodium azide added in 1953 had by 1954, lost its depressing effect on growth, and yields were good. However, other effects of its presence are apparent in the results. Firstly, the yields were approximately constant throughout, and secondly, the concentrations of Mn were again greater in plants grown at pH 7 than at pH 8. Another effect of sodium azide was to increase markedly the concentrations of Mn in plants growing in soil to which it had been added. Indeed, the concentrations in the oat straw of 1953 were extraordinarily high, and although they decreased substantially in 1954, they were still much higher than those in plants grown in the non-sterilized soil.

CONCLUSIONS

The pot experiments have shown that liming the neutral Penola soil to pH 8 had the following effects on oats. Firstly, it increased

the concentration and uptake of Mn and secondly, it increased the yields of dry matter where Mn in the soil was apparently limiting. When the soil was initially sterilized with sodium azide, liming had the opposite effect on Mn in the plants, and at the higher pH both the concentration and uptake of Mn were decreased. Mn deficiency was overcome by sterilizing, and liming then had no effect on yields. This means that the Mn in the sterilized soil was at no point limiting growth.

In earlier experiments [3] using Penola soil it has been shown that sodium azide stabilized bivalent Mn, presumably by inhibiting the Mn-oxidizing micro-organisms. Further, the effect of liming the sterilized soil to pH 8 was to decrease the amount of reduction of added δ-MnO_2 to bivalent Mn. This is as expected, since MnO_2 becomes a weaker oxidizing agent with increasing pH. These effects are compatible with the results of the present pot experiments with sterilized Penola soil, and lead to the explanations, firstly, that Mn deficiency was overcome by stabilizing bivalent Mn at an unusually high level, and secondly, that Mn was less available at pH 8 than at pH 7 because the extent of reduction to bivalent Mn was less at pH 8.

The greater uptake at pH 8 found in the unsterilized soil could be due to greater quantities of bivalent Mn existing at this higher pH. Mulder and Gerretsen [10] after Mattson [9] suggest that bivalent Mn could accumulate by the production of more active reducing agents in the course of the autoxidation of the organic matter after liming while Leeper and Swaby [6] suggest that bivalent Mn could be removed from the oxidation cycle as $MnCO_3$ which is available to plants. The often quoted statement of Lundegardh [7] that Mn becomes more soluble at pH 8 or higher does not help and seems to rest on a misunderstanding. He did not report any experiments in raising the pH of a soil above 8, but merely recorded the fact that none of the four Swedish soils of pH over 8 which he collected produced Grey-speck disease of oats. As he himself remarks soils of pH 8 and above are rare in Sweden.

SUMMARY

The uptake of manganese by oats in a manganese-deficient soil was increased on raising the pH from 7.0 to 8.0 with $Ca(OH)_2$. This effect occurred in the absence of added manganese as well as when manganese was added to

the soil as δ-MnO_2. When the soil was initially sterilized the uptake of manganese at pH 7.0 was greatly increased at both pH's, but the uptake at pH 8.0 was now a smaller than at pH 7.0.

ACKNOWLEDGEMENTS

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THE EFFECT OF LIMING A NEUTRAL SOIL ON THE CYCLE OF MANGANESE

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INTRODUCTION

The uptake of manganese by oats in a manganese-deficient clay (from Penola, South Australia) has been increased on raising the pH from 7.0 to 8.0 with calcium hydroxide [9]. This effect occurred in the absence of added manganese as well as when 10, 18, 32 and 60 parts of manganese per million (ppm) were added to the soils as δ-MnO_2. This is a surprising result, because in general we find the opposite relation between pH and the uptake of manganese by plants. The expected relation did, however, hold if the soil was initially sterilized with sodium azide, when the uptake of manganese was decreased on raising the pH from 7.0 to 8.0. The increased uptake of manganese which occurred on liming the unsterilized soil led to the present studies of the effect of lime on the reactions of manganese in the soil.

EXPERIMENTS

The first line of attack was to determine the bivalent Mn in unsterilized Penola soil from the pot experiments already described [9] using as extractants solutions of ammonium acetate alone, and ammonium acetate containing disodium calcium ethylenediamine tetra acetate (EDTA solution). The use of this last solution follows the work of Beckwith [1] which is discussed more fully later (page 321).

During March 1955, samples were taken from the pots of soil which contained 60 ppm of added Mn. These were extracted after they had been air dried for 8 days. For the ammonium acetate extraction 100-g samples of the

soil were shaken mechanically for 2 hours with 1,000 ml of molar ammonium acetate. Stock solutions of ammonium acetate were adjusted to pH 7.0.

The EDTA solution was prepared by mixing equal volumes of a solution of 2 *M* ammonium acetate (adjusted to pH 7.0) with a solution containing 2% of disodium calcium ethylenediamine tetra acetate, the calcium salt having been prepared previously by saturating the disodium dihydrogen salt with $CaCO_3$ and filtering. Soil samples of 30 g were shaken mechanically for 2 hours with 300 ml of EDTA solution. The pH's of suspensions containing the limed soils were adjusted with acetic acid to give the same pH's as those given by the unlimed soils.

After being shaken the suspensions were centrifuged and filtered. The organic matter in the filtrates was destroyed by alternate treatments with hydrogen peroxide and aqua regia and finally the Mn was estimated by the colorimetric–periodate method using a Hilger Spekker absorptiometer.

The ammonium acetate and EDTA solutions extracted 0.85 and 3.56 ppm of Mn respectively from soil with pH 8.0, and 0.18 and 1.21 ppm respectively from soil with pH 7.0. These results suggest that liming affects the oxidation—reduction cycle of Mn in this soil. Further experiments were therefore undertaken to examine the effect of lime on both the reduction and oxidation of Mn.

Release of bivalent manganese from MnO_2

The experiments on reduction consisted essentially of estimating the release of bivalent manganese from a synthetic manganese dioxide when mixed with Penola soil at pH's 7.0 and 8.0. This manganese dioxide was from the same batch of synthetic δ-MnO_2 as was used in the pot experiments [9].

A bulk sample of the unlimed Penola soil was collected moist from an unsown pot. After air-drying for 20 hours, in order to allow easy grinding and mixing, the soil was ground to pass a 2-mm sieve. Six sub-samples of 1,000 g each were taken and three of these were limed to pH 8.0 by intimately mixing 3.125 g $Ca(OH)_2$ into each. Each sample of soil, in a weighed beaker with a stirring rod, was mixed with 100 ml of water and placed in an incubator at 20°C. By adding water daily all samples were kept at constant weight for four days and then were removed from their beakers and spread onto polythene sheeting. The MnO_2 was added to both limed and unlimed samples at the rates of 60, 120 and 240 parts of Mn per million of the air-dry soil. The soil mixtures were returned to their respective beakers and were brought to 40% of their water-holding capacity and incubated at 20°C. By adding water to constant weight and stirring every day the soils were kept constantly and uniformly moist throughout the incubation period.

In a smaller-scale experiment δ-MnO_2 was added to air-dried Penola soil

The air-dried soil was taken from a glass jar in the laboratory where it had been stored for 6 months. After grinding to pass a 2-mm sieve, two samples of 120 g each were taken and one of these was limed to pH 8.0 by mixing with 0.375 g $Ca(OH)_2$. Each sample, in a weighed beaker with stirring rod, was placed in the incubator at 20°C. On the fourth day MnO_2 was thoroughly mixed with each at the rate of 120 parts of Mn per million of soil.

At various intervals after mixing MnO_2 with the soils, sub-samples were removed and extracted for bivalent Mn using ammonium acetate and EDTA solutions. The methods were as described above with two exceptions; the moist soils were extracted immediately without a preliminary air-drying and the pH's of the suspensions containing the limed soils were not adjusted to those given by the suspensions of unlimed soils.

The moist soils containing 60 ppm of added Mn were extracted at frequent intervals up to the 32nd day after adding MnO_2, and the progressive release of bivalent Mn during this period is shown in Fig. 1. The results show that greater amounts of bivalent Mn were extracted at pH 8 than at pH 7. While the difference between the

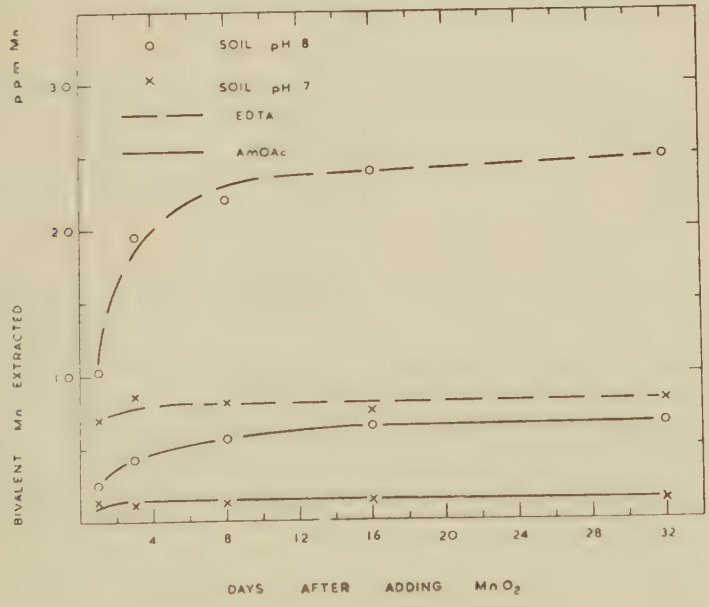

Fig. 1. The effect of raising the pH on the release of bivalent Mn from mixtures of moist soil with 60 ppm Mn added as δ-MnO_2.

amounts of Mn extracted from the two soil mixtures at one day was slight, it thereafter became marked, since the bivalent Mn increased steadily in the soil mixture with pH 8 but only slightly at pH 7.

The other moist soils, containing 120 and 240 ppm of added Mn, were extracted at 32 days after adding the MnO_2. The results (Table I) show greater amounts of bivalent Mn at pH 8 than at pH 7 at each level of added MnO_2 and a linear relation between the amounts of MnO_2 added and bivalent Mn extracted.

TABLE I

Soil pH	Bivalent Mn extracted on the 32nd day of incubation of δ-MnO_2 with moist Penola soil (ppm Mn)					
	Added Mn 60 ppm		Added Mn 120 ppm		Added Mn 240 ppm	
	AmOAc	EDTA	AmOAc	EDTA	AmOAc	EDTA
8.0	0.66	2.49	0.83	3.77	1.12	6.67
7.0	0.13	0.82	0.15	0.87	0.16	1.03

The air-dried soils were extracted at 16 and 32 days after adding MnO_2 and the results (Table II) show that greater amounts of bivalent Mn were extracted at pH 7 than at pH 8. This is in striking contrast to the results obtained with the moist soils and led to a

TABLE II

Soil pH	Bivalent Mn extracted from mixtures of δ-MnO_2 with air-dry Penola soil (120 ppm Mn added) (ppm Mn)			
	16 days		32 days	
	AmOAc	EDTA	AmOAc	EDTA
8.0		4.5	1.6	7.0
7.0		9.5	4.7	14.6

further experiment on the release of bivalent Mn in air-dried soils. For this further experiment the moist soils which were used previously were allowed to dry and after remaining so for 10 months they were extracted for bivalent Mn. The results (Table III) confirm the observation that greater amounts of bivalent Mn are released at pH 7 than at pH 8 in air-dried soils and they show also that there is a linear relation between the amounts of added MnO_2 and

TABLE III

Soil pH	Bivalent Mn extracted from mixtures of δ-MnO_2 and Penola Soil which were initially moist but subsequently air dried for ten months (ppm Mn)					
	Added Mn 60 ppm		Added Mn 120 ppm		Added Mn 240 ppm	
	AmOAc	EDTA	AmOAc	EDTA	AmOAc	EDTA
8.0	5.6	20.3	11.9	40.4	12.4	45.4
7.0	11.6	35.2	20.8	61.5	35.5	104.2

bivalent Mn extracted. These opposite findings with air-dry and moist soils will be found to fit the results of the next experiments.

Oxidation of bivalent Mn

A bulk sample of unlimed Penola soil was collected moist and after air-drying for 20 hours it was ground to pass a 2-mm sieve. Two sub-samples of 200 g each were taken and one of these was limed to pH 8 by mixing with 0.625 g $Ca(OH)_2$. Each sample, in a weighed beaker with stirring rod, was mixed with 20 ml of water and placed in the incubator at 20°C where it was kept at constant weight for four days. On the fourth day a 20-ml aliquot of a solution of $MnSO_4$ was thoroughly mixed into each sample to give a concentration of 60 parts of manganese per million of the soil (air-dry basis). The samples were then returned to the incubator and by adding water to constant weight and stirring each day they were kept constantly and uniformly moist throughout the incubation period.

In a parallel experiment air-dried Penola soil was used, two samples of 100 g each being taken from the batch which had been stored for 6 months. One sample was limed to pH 8.0 by mixing with 0.3127 g $Ca(OH)_2$ and then both were placed in the incubator at 20°C in beakers as before. After four days incubation a 10-ml aliquot of a solution of $MnSO_4$ was thoroughly mixed into each sample to give a concentration of 60 parts of Mn per million of the air-dry soil. The samples were then returned to the incubator and allowed to dry.

At various intervals after adding the solutions of $MnSO_4$ to the soils, sub-sampled were removed and extracted for bivalent Mn using ammonium acetate and EDTA solutions. The methods were as described for the experiments on the release of bivalent Mn from MnO_2.

The results of extractions of the moist soils are presented as curves in Figs. 2 and 3 which reflect progressive oxidation. They show that the rate of oxidation is less at pH 8 than at pH 7 and also that the final level of bivalent Mn is higher at pH 8 than at pH 7. On the other hand the amounts of bivalent Mn extracted from the air-dried soils remained practically constant even up to 10 months after adding $MnSO_4$ (Table IV). The recovery of the added

TABLE IV

Soil pH	Bivalent Mn extracted from mixtures of $MnSO_4$ with air-dry Penola soil (60 ppm Mn added) (ppm Mn)					
	2 days		32 days		10 months	
	AmOAc	EDTA	AmOAc	EDTA	AmOAc	EDTA
8.0	21	52	19	47		46
7.0	28	56	23	51		49

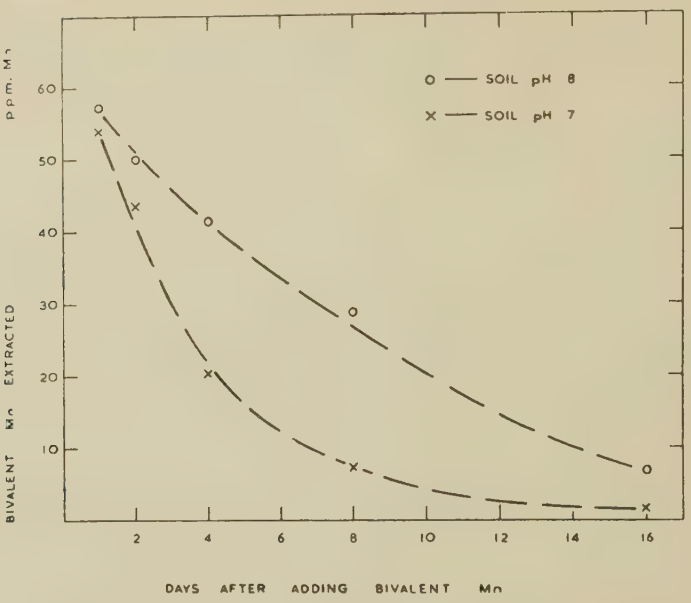

Fig. 2. The effect of raising the pH on the rate of oxidation of bivalent Mn in moist soil; 60 ppm Mn added as $MnSO_4$. Extractant: EDTA solution

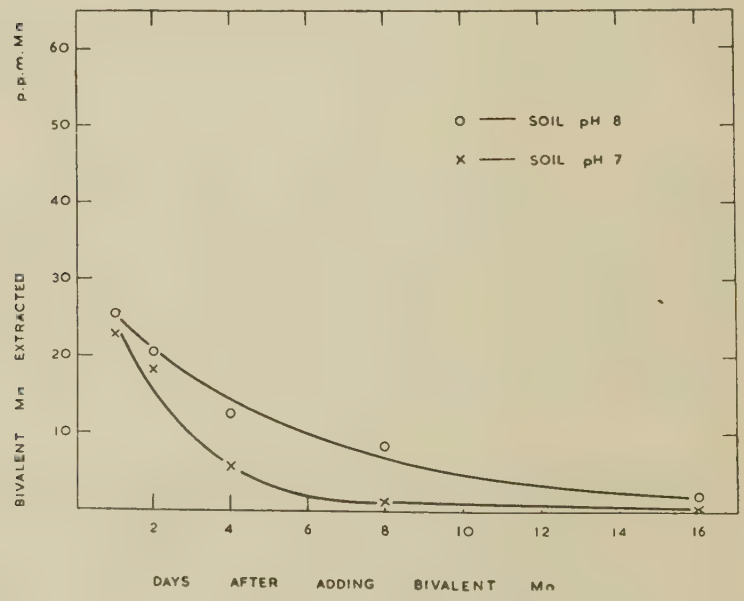

Fig. 3. The effect of raising the pH on the rate of oxidation of bivalent Mn in moist soil; 60 ppm Mn added as $MnSO_4$. Extractant: Ammonium acetate.

bivalent Mn with EDTA solution was practically complete, which shows that oxidation was stopped in the air-dried soils.

DISCUSSION

An important part of the present study consisted of tracing the fate of manganese compounds added to the Penola soil. This was done by estimating the bivalent Mn which was either produced by reduction after MnO_2 was added to the soil, or left unoxidized when $MnSO_4$ was added. The usual way of estimating bivalent Mn in soils is to extract with neutral electrolytes such as ammonium acetate, calcium nitrate and magnesium nitrate. There are, however, disadvantages with electrolytes since their efficiency varies, depending on the replacing cation, the pH of the suspension, and the time of extraction. Recently Beckwith [1] has found a highly efficient extractant in the combination of disodium calcium ethylenediamine tetra acetate (EDTA) and calcium acetate. Since errors could arise through the inefficient extraction of bivalent Mn it seemed desirable to use EDTA in the present work. The EDTA was used in a combination with ammonium acetate (not calcium acetate) and some estimate of its efficiency was obtained by extracting separately with ammonium acetate alone.

The relative amounts extracted by the two reagents varied but in most cases EDTA solutions extracted between three and six times as much Mn as did ammonium acetate. Since bivalent Mn can be very firmly held by some colloids, especially by some organic colloids [2 8] it is not surprising that a strong chelator is needed to remove the most firmly held fraction.

Although several cycles of Mn in soils have been suggested [4 5], the only well established changes, besides the aging and weathering of higher oxides, are those of reduction and oxidation, about which the following general statements may be made. Manganese oxides are reduced to bivalent Mn by organic matter, the more readily the lower the pH of the soil [12 13]. The opposite reaction of oxidation is due to micro-organisms [7 14 17]. It occurs in well-aerated soils with pH's above 5.5, but is very rapid between pH's 6.0 and 7.5 [14]. Thus, the amounts of bivalent Mn are small in soils with pH's above 6.0 since oxidation is rapid and reduction becomes progressively slower.

Air-dry soils

The effects of air-drying may first be considered. Since added bivalent Mn is completely recovered from air-dried soil (Table IV) it must be concluded that the Mn-oxidizing micro-organisms are completely inhibited. There is further evidence of inhibited oxidation in the very large increases of bivalent Mn which occurred when the moist Penola soils containing MnO_2 were dried (Tables I and III). In these soils the only reaction would be the reduction of MnO_2 by organic matter; hence the bivalent Mn would accumulate. Several workers [3 6 19] have observed a slow release of bivalent Mn in air-dry soils. This slow reduction might well be through a slowly volatile component of the organic matter. It could hardly be microbial in such a dry state.

The air-dried Penola soils may be regarded as non-biological systems in which the rate of reduction of MnO_2 would depend on the difference between its redox potential and that of the soil, and this would decrease with increasing pH. This relation is reflected in a general way in the amounts of extracted bivalent Mn, which were invariably greater at pH 7 than at pH 8 although theoretically one might have expected an even greater difference than was found. Similar relations were found previously [10] when moist Penola soil was sterilized with sodium azide and incubated in mixtures with MnO_2.

In non-biological systems at constant pH, the final concentration of bivalent Mn would depend on the amount of MnO_2 added. It may be assumed that in the air-dried soils, the bivalent Mn has in ten months approached final concentrations. At ten months a linear relation was found at pH 7 between the amounts of MnO_2 added and bivalent Mn extracted. There was at pH 8 a similar relation where 60 and 120 ppm Mn was added, but when 240 ppm Mn was added the bivalent Mn barely increased (Table III). This indicates that the highest level of MnO_2 provided a surplus of oxidant over available reductant. It follows from this that there would be a critical level or active mass of MnO_2 at any pH, and if this is exceeded the linear relation to bivalent Mn will no longer hold.

Moist soils

In the moist Penola soils the amounts of bivalent Mn are of a

much lower order and again these have to be considered in terms of the opposing reactions of oxidation and reduction. It has been shown in the present work that added bivalent Mn is rapidly oxidized at pH 7 whereas it is slowly oxidized at pH 6 (Figs. 2 and 3). This is compatible with results obtained by Gerretsen[7] and by Leeper and Swaby[18] who found that rings of manganese oxide appeared rapidly in soil agar plaques with central plugs of $MnSO_4$ near when pH's were between 6.2 and 7.2. At pH's above and below this range the rings of manganese oxide took longer to appear. The slower oxidation below pH 6.2 may be explained both by microbial inhibition and by the quicker reduction of manganese oxide once formed. The slower oxidation at pH 8.0 in Perola soil may similarly be explained by microbial inhibition as suggested by Gerretsen[7] and also by decreased mobility of bivalent Mn.

In most Perola soils to which 60 ppm of Mn was added as MnO_2 the bivalent Mn had reached apparent equilibrium concentrations in 32 days (Fig. 1). It is noticeable that at pH 7 there was no difference between the amounts of bivalent Mn found at 1 and 32 days after adding the MnO_2. This indicates that the added Mn gets rapidly into the oxidation—reduction cycle. Thus while MnO_2 is reduced, the life of the bivalent Mn so produced is short because of rapid oxidation. At pH 6 an equilibrium is reached more slowly presumably because both reduction and oxidation are slower. Apparently the rate of oxidation is lowered sufficiently to give an increase in bivalent Mn so that the amounts exceed those at pH 7.

The moist soils containing 120 and 240 ppm of Mn added as MnO_2 were extracted at 32 days and it is reasonable to assume that by that time the amounts of bivalent Mn were approaching equilibrium concentrations. The increases in bivalent Mn produced by increasing amounts of MnO_2 are much greater and more definite at pH 6 than at pH 7 (Table 1). These relatively large increases are apparently characteristic of soils in which microbial oxidation is inhibited.

Availability of Mn to plants

The studies of the oxidation—reduction cycle of Mn lead finally to a discussion of the availability of Mn to oat plants in the Perola soils. The δ-MnO_2 used in both pot experiments[5] and incubation experiments is highly active and has a large surface area. Added in increasing amounts to the soil this MnO_2 produced increases in the

uptake of Mn by oats at both pH's 7 and 8. This relation held for plants grown in the year in which the MnO_2 was added (1953) and in the following year as well. In the absence of figures for the bivalent Mn which actually existed in the soils while the oats were growing it is possible to extrapolate from the results of the present incubation experiments and so make estimates of the likely amounts of bivalent Mn. A comparison may then be made between the added MnO_2, the expected bivalent Mn in the soil, and the Mn taken up by the plants in the pot experiments of 1953 (Table V).

TABLE V

Soil pH	MnO_2 ppm	Bivalent Mn * ppm	'Influential Mn' ** ppm	Plant uptake † μg
7.0	18	0.79	1.15	510
	32	0.80	1.44	700
	60	0.82	2.02	1,060
8.0	18	1.52	1.70	950
	32	1.83	2.15	1,120
	60	2.49	3.09	1,520

* Bivalent Mn as found by extrapolation from Table I.

** 'Influential Mn' at pH 7 : Bivalent Mn $+ \frac{MnO_2}{50}$

at pH 8 : Bivalent Mn $+ \frac{MnO_2}{100}$.

† Plant uptake: Total Mn absorbed by oat plants in 1953 (Jones[9], Fig. 5).

In the range of 18 to 60 ppm of added Mn the relative increase in uptake by oats at pH 7 was greater than that reflected by the relative increase in bivalent Mn in the soil. The only possible explanation for this discrepancy lies in the increasing amounts of MnO_2. The MnO_2 consists of individual grains and doubling the amount added would be equivalent to doubling the active surface. In turn this would mean doubling the area of contact of plant root with MnO_2. The root probably obtains its Mn directly from the surface of the MnO_2 but the mechanism is unknown. A number of synthetic oxides with composition Mn_2O_3 to MnO_2 have previously been found to supply oats adequately with Mn and furthermore, there is evidence to suggest that their ability to do so depends on their high activity and surface area [11]. For the purposes of the present discussion it is only possible to make a speculative allowance for the direct supply of Mn from MnO_2 to the root. Estimates of a

fraction referred to as 'influential Mn' can be obtained by making, say, $^1/_{50}$ of the added MnO_2 equivalent to bivalent Mn and then adding this to the bivalent Mn present. By taking the two sources of Mn into account at pH 7 a closer relation is found to the Mn taken up by plants.

The uptake of Mn at pH 8 can be accounted for by the bivalent Mn in the soil but to be consistent some allowance should be made for the direct supply of Mn from MnO_2 to the root. To allow for the higher pH of the soil, $^1/_{100}$ of the added MnO_2 may be made equivalent to bivalent Mn. The amounts of 'influential Mn' obtained are only slightly greater than those of bivalent Mn. The main effect of taking MnO_2 into account is at pH 7 where the amounts of bivalent Mn appear inadequate. The nett result is that the amounts of 'influential Mn' at pH 7 bear a constant relation to those at pH 8 and this is in agreement with the relation between the uptake by plants at pH 7 and pH 8.

The preceding speculation does not affect the conclusion that the greater uptake by plants at pH 8 is due to greater amounts of bivalent Mn existing at each level of MnO_2. The effect of $Ca(OH)_2$ is somewhat analogous to that of sterilizing agents in that oxidation is inhibited, thereby allowing the concentration of bivalent Mn to increase.

The improvement of a deficient soil by liming to pH 8 may seem inconsistent with the facts that raising the pH has lowered the amounts of bivalent Mn in the Danish soils reported by Steenbjerg [18] and that many soils of pH 8 show acute manganese deficiency. We should expect the rates of both oxidation and reduction to be lower at the higher pH but if oxidation is retarded more than reduction the results will be as described for the Penola soil. What seems striking about this soil is that the rate of reduction is decreased by no more than 60 to 70 per cent, whereas one might have predicted a decrease of 99 per cent for a tenfold decrease of hydrogen ion concentration as given by the reaction:

$$MnO_2 + 2H^+ + C_6H_4(OH)_2 \rightarrow Mn^{++} + 2H_2O + C_2H_4O_2.$$

This suggests that the reducing groups responsible for the reduction at pH 8 are not identical with those responsible at pH 7, but that fresh and more active reducing compounds are produced as intermediate products of autoxidation as suggested by Mattson,

Eriksson and Vahtras [16]. The Penola soil comes from a former swamp [9] and its organic matter may therefore have unusual properties. If the production of these compounds is only temporary this could explain the decline of the beneficial effect of $Ca(OH)_2$ with time as reported elsewhere [15].

SUMMARY

A manganese-deficient soil was mixed at pH 7 and pH 8, both moist and dry with (*a*) δ-MnO_2 and (*b*) $MnSO_4$. The amounts of bivalent manganese were determined after incubating for varying times. With the moist soils more bivalent manganese was released at pH 8 than at pH 7. However, after long air-drying much more bivalent manganese was released than from the moist soils and more was released at pH 7 than at pH 8.

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THE RELATIVE CONTENT OF MANGANESE IN PLANTS

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INTRODUCTION

Two of the most interesting problems about the uptake of micronutrient elements by plants are first, the relative concentrations which are produced by increasing the amount of an element which is in short supply and second, the differing abilities of plants to absorb an element from the same source. Both of these themes are illustrated in pot experiments here described on a manganese-deficient soil.

The experiments consisted essentially of comparing the uptake of manganese by different plants from increasing additions to the soil of a synthetic δ-MnO_2. This higher oxide of manganese was chosen as a source of manganese rather than manganese sulphate for two reasons. Firstly, it was assumed from earlier experiments [6] that δ-MnO_2 was as good as $MnSO_4$ at supplying plants with manganese. Secondly, a known higher oxide is more satisfactory since it is stable for some months at least whereas added bivalent manganese would be rapidly converted to an unknown higher oxide [3 8] while a small fraction may also form complexes with organic matter [2 4].

The following plants were grown as successive crops: *Avena sterilis* ("Algerian" variety); *Vicia sativa* (commercial variety "Golden Tares") and *Secale cereale* (commercial variety "South Australia"). These plants were chosen because of their differing susceptibilities to manganese deficiency. Oats are highly susceptible, rye is much less so [7 12] and vetch is not susceptible.

The soil selected for these experiments was collected from the field at Rendelsham in the south-east of South Australia in January,

1953. It is a grey, highly calcareous peat of pH 7.9 and it contains 4.8 parts per million of manganese extractable with ammonium acetate containing quinol. This soil is similar to one used previously by Jones and Leeper [6] but it differs from the black manganese deficient peat of pH 6.5 from Rendelsham which has been used by Beckwith [1] and which is not calcareous.

METHODS

The experiments were started in June 1953, when oats were sown; they were continued by resowing the same pots with vetch in 1954 and with rye in 1955. The details where different for the three crops will be described separately.

The δ-MnO_2 was from the same batch as was used in the pot experiments described in an earlier paper [5]; it was precipitated from a solution of $Ca(MnO_4)_2$ by adding a solution of $MnSO_4$.

The culture pots were of glazed earthernware and held 4.3 kg of soil. Before mixing the soil was air dried and broken down to pass a 3-mm sieve. The δ-MnO_2 was intimately mixed throughout each 4.3 kg sample to give pots containing 0, 10, 18, 32, 60, 100 and 180 parts of manganese per million of the soil. There were two pots of soil at each level of manganese.

Superphosphate was mixed at the rate of 1.57 g throughout each pot of soil, which then received a surface application of the following: KNO_3, 630 mg; $MgSO_4.7H_2O$, 157 mg; $ZnSO_4.7H_2O$, 14 mg; and $CuSO_4.5H_2O$, 14 mg. This surface application was repeated, together with one of 1.00 g of Na_2HPO_4 per pot, before sowing vetch and again before sowing rye. All reagents were free of manganese.

The pots of soil were brought to 60% of the water-holding capacity (*i.e.* total pore space) and oats were sown in mid-June, 1953. The plants were grown to maturity and harvested in mid-December, 1953. Vetch was grown during the same months of the following year (1954) whereas rye was sown in early March, 1955, and harvested in late April, 1955. The plants were grown in the open in a birdproof enclosure in the grounds of the Agriculture School, Melbourne University, and were protected from rain by a rolling tarpaulin. Pots were weighed at two-day intervals and watered by hand in an attempt to keep the soil at 60% water-holding capacity. Seeds and straw were separated, dried at 105°C for 15 hours and weighed. Samples of the seed and straw were digested with a mixture of perchloric, nitric and sulphuric acids and manganese was then estimated by the colorimetric-periodate method using a Hilger 'Spekker' absorptiometer.

RESULTS

Oats

The oats showed severe symptoms of manganese deficiency where no manganese was added to the soil. With increasing additions

the symptoms became progressively less severe until with 32 ppm of added manganese they disappeared completely (Plate Ia). The yields of straw increased from 1.3 g to 22.5 g with 32 ppm of added manganese. No grain was produced with 0 and 10 ppm of manganese added to the soil but the yield increased from 2.5 g with 18 ppm of added manganese to 10.6 g with 32 ppm of added manganese. Additional manganese up to 180 ppm caused no further increase of straw or grain. In a less deficient soil from Penola only 10 ppm of manganese as δ-MnO_2 was needed to overcome deficiency [5]. Taken together these results show that δ-MnO_2 provides an effective source of manganese for plants.

The concentration of manganese in the straw and grain is shown in Fig. 1. A striking feature of each curve is the sharp minimum in

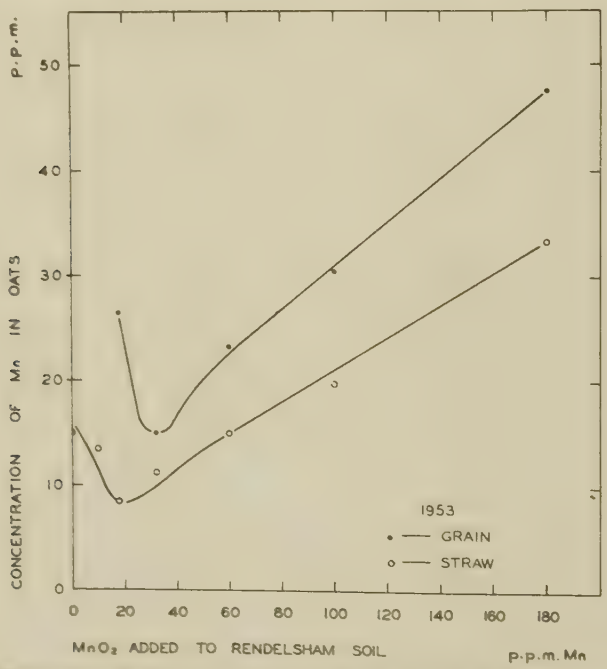

Fig. 1. Relationship between amount of MnO_2 added to Rendelsham soil and concentration of manganese in oats.

concentration produced by the first additions of manganese. This is particularly striking for the grain in which the minimum concentration occurs at the very point at which additional manganese in

the soil gives no increase in yields (Fig. 2). A similar effect to this has been found by Steenbjerg [13 14] for copper. With increasing additions of copper compounds to a soil, the concentration in

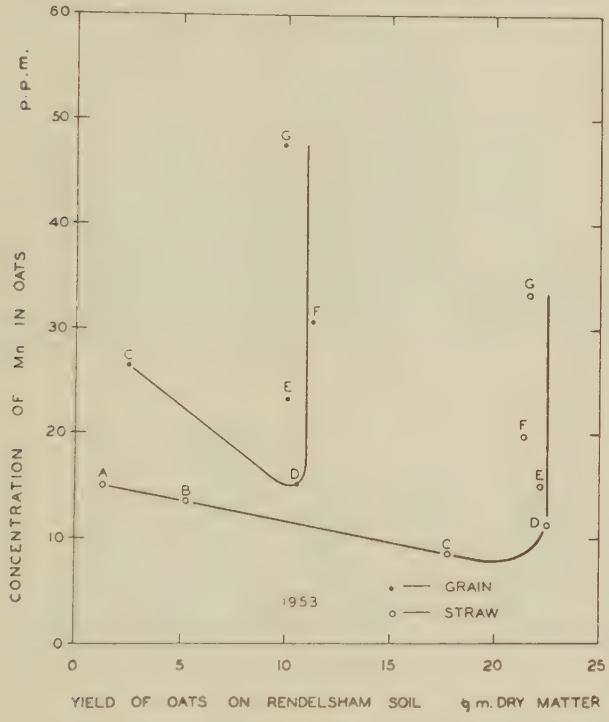

Fig. 2. Relationship between yield of dry matter and concentration of manganese in oats. Parts of manganese per million of soil added as δ-MnO_2: A = none, B = 10, C = 18, D = 32, E = 60, F = 100, G = 180.

barley plants fell to about half the value of the control, and rose only after the maximum yield had been attained. The results obtained for manganese in the present study repeat this last relation exactly. Two earlier contributions [10 11] on copper which illustrate a similar minimum have not, as yet, been widely noticed.

Some unpublished work by Stout and Pearson quoted by Ulrich [15] is of interest here. When zinc was in limiting supply in culture solutions they found that it accumulated in the growing points and nodes of their experimental maize plants but not in the internodes. The first additions of zinc to the cultures produced

increased growth and a parallel decrease in concentration of zinc in the entire tops. This decrease in concentration in the tops was explained by the fact that most of the increased growth was due to an increase in the proportion of internodal tissue. It is possible that a similar explanation holds for the observed results with manganese and copper.

Vetch and rye

Vetch showed no symptoms of manganese deficiency and its growth was uniformly good (Plate Ib). Rye likewise showed no symptoms of manganese deficiency and although its growth was uniform it was restricted by its short growing season (Plate Ic).

The concentrations of manganese in the straw of both vetch and rye are shown in Fig. 3. Instead of a minimum as found in oats, the

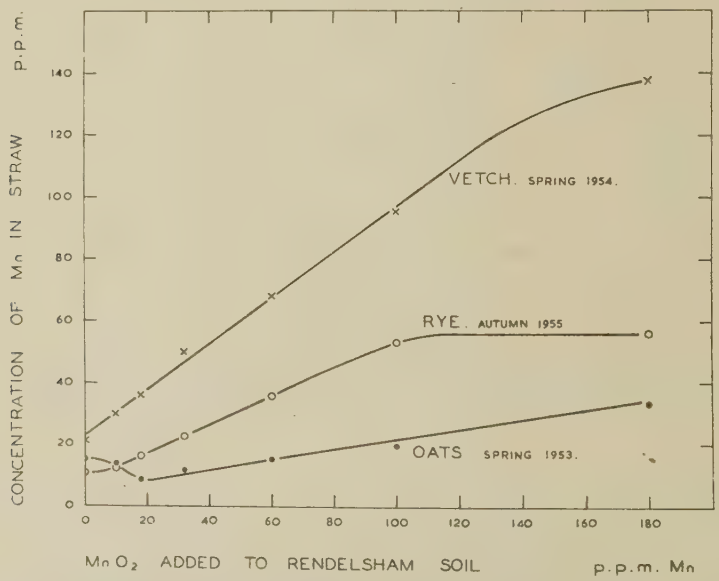

Fig. 3. Relationship between amount of MnO_2 added to Rendelsham soil and concentration of manganese in oats, rye, and vetch.

concentrations increased with increasing manganese in the soil. The ability of the vetch to obtain manganese is outstanding and even with no manganese added to the soil the concentration of manganese in it exceeded those in both rye and oats. With in-

Plate I. **A.** Oats on Rendelsham soil. Left to right: control, 10, 18, 32, 60. 100, 180 ppm of manganese added as δ-MnO_2. **B.** Vetch. Soil and manganese additions as for oats. **C.** Rye on Rendelsham soil. Left to right: control, 10, 18, 32, 60, 100 ppm of manganese added as δ-MnO_2.

creasing additions of manganese to the soil vetch concentrated manganese to a much greater degree than did oats; rye was intermediate between the two.

If the yields are taken into account, and plotted against concentrations (see Fig. 4) a striking contrast is obtained between the curves for oats on the one hand and vetch and rye on the other.

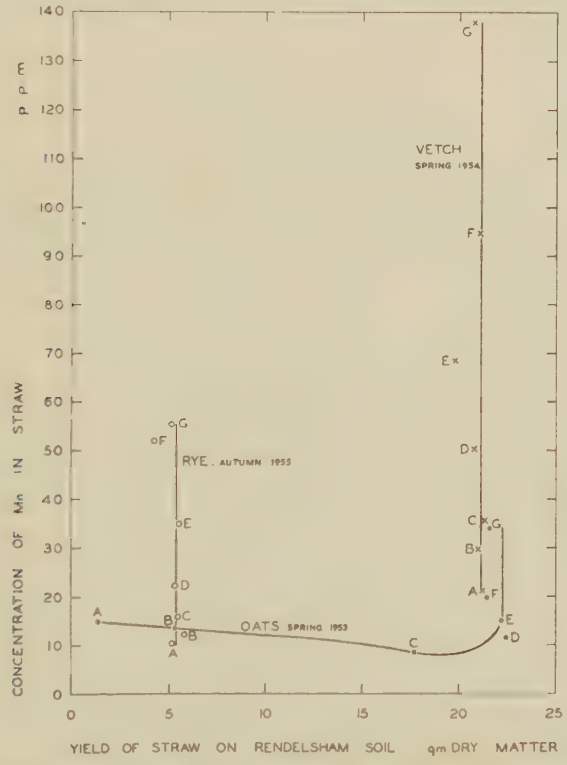

Fig. 4. Relationship between yields of dry matter and concentrations of manganese in oats, rye and vetch. Parts of manganese per million of soil added as δ-MnO_2: A = none, B = 10, C = 18, D = 32, E = 60, F = 100, G = 180.

The increasing yields of oats produced by the first additions of MnO_2 to the soil are accompanied by decreasing concentrations of manganese in the plants. When the yield of oats is no longer affected by increasing manganese in the soil the curve becomes similar to those given by vetch and rye. The contrast between oats

and vetch is also illustrated by the total amounts of manganese absorbed per pot (Fig. 5).

The outstanding ability of vetch to obtain manganese from an alkaline soil can be set against the large uptake from acidic soils

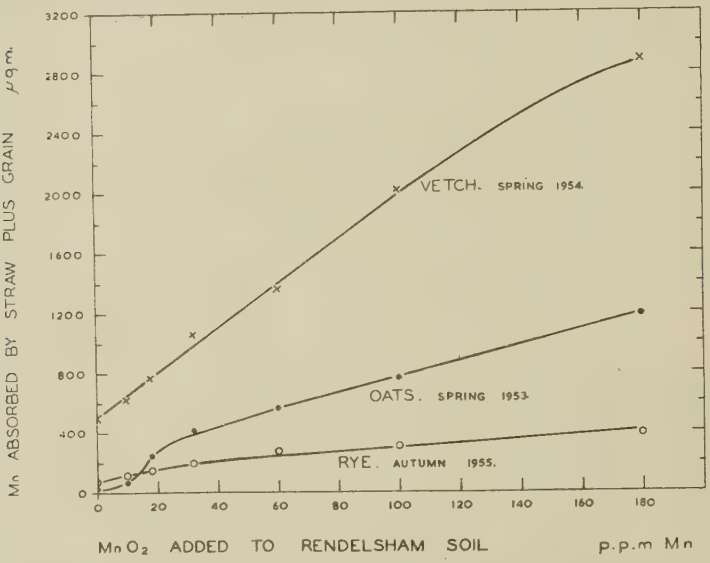

Fig. 5. Relationship between amount of MnO_2 added to Rendelsham soil and manganese absorbed by oats, rye and vetch.

in which manganese is present largely as the bivalent ion. For example, in an acidic soil in Holland, vetch accumulated about 2,500 ppm of manganese at which concentrations it suffered from excess manganese. Oats on the other hand were unaffected by excess manganese and accumulated only 370 ppm [9]. Similarly, vetch took up four times as much manganese as oats on the alkaline soil. However, it must be emphasized that both plants must have different mechanisms for obtaining manganese when bivalent manganese and MnO_2 are the sources. The mechanism by which manganese is taken up from MnO_2 by the plant root is unknown. The different abilities of vetch and oats may be due to different absorbing mechanisms at the surface of the root, or different rhizosphere flora or different root excretions.

SUMMARY

Oats, vetch, and rye were grown as successive crops in pots of a manganese-deficient soil to which δ-MnO_2 had been added in increasing amounts. Manganese deficiency of oats was overcome with 32 ppm of added manganese. Additions of 10, 18 and 32 ppm caused a decrease in concentration of maganese in the plants. Vetch and rye were unaffected by manganese deficiency and kept on accumulating manganese with increasing additions to the soil. Of the three plants vetch showed the greatest uptake of manganese, both with and without added manganese.

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COMPARATIVE CHLORINE REQUIREMENTS OF DIFFERENT PLANT SPECIES

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INTRODUCTION

Since our earlier report [1] that chlorine is a micronutrient element as shown in experiments with the tomato plant, ten other species have been grown under similar cultural conditions. Within the series tested, during a winter season, severe chlorine-deficiency symptoms were produced with lettuce, tomato, cabbage, carrot, sugar beet, barley, and alfalfa. Yield effects, but not obvious symptoms of disease, were obtained with buckwheat, corn, and beans, whereas squash showed no effect in yield and only suggestions of nutritional deficiency symptoms. These wide differences in effect of low chlorine supplies are characteristic of the individual species since replicated causal conditions within a species have always resulted in equivalent plant effects.

Of the species tested thus far, the lettuce plant has proven to be the most sensitive to low chlorine supply. By contrast, squash plants cultivated at the same time under the same conditions were scarcely affected. Chemical analyses of plant materials cultured on low-chlorine solutions have shown the presence of considerably more chlorine than the amounts which were known to be provided in their culture solutions. Moreover, species least affected by low chlorine supplies appeared to be favored in their abilities to acquire chlorine from extrinsic * sources — undoubtedly the atmosphere.

* In the work, two types of contamination are recognized. The first, *intrinsic* to the plant—culture system, includes known contamination from seed, in the repurified salts and water, and from aeration. The second, *extrinsic* to the system, includes that from airborne or other sources unknown or uncontrolled.

Mechanisms by which some species are able to acquire more extrinsic chlorine than others are the subject of further study. It is suspected that leaf structures of different types of plants are involved and that these organs capture airborne chlorine. Our greenhouses are located in a heavily populated area near the sea. For these reasons, aerosols from sea spray and a variety of chlorine compounds originating from industrial and domestic furnaces are constantly in the atmosphere. The amount of leaf spread characteristic of different species is suggestive though inadequate to explain the great differences in the deficiency susceptibility of different species to the same levels of low chlorine in culture solutions. For example, the highly affected lettuce plant has leaves formed into a compact head whereas leaves of squash plants spread into an open canopy.

From the present experiments, it appears that chlorine is a requirement for the nutrition of higher plants in general, but its demonstration presents greater experimental difficulties for some species than for others.

MATERIALS AND METHODS

The composition of the culture solutions is given in Table I. Amounts of specific salts supplied are given in micromoles per liter. The thirteen elements (now including chlorine) which must be added to solution cultures are listed in microgram atoms per liter in descending order of concentration on the right hand side of the table. The complete culture solution containing the thirteen elements is designated M6 + m7. Concentrations of the macronutrient salts (Solution M6) are little different from those of other standard culture solutions. Concentrations of the micronutrients in Solution m7 are reported in micromoles per liter because, in laboratory practice, all stock solutions are prepared on a chemical equivalent basis. Among the micronutrients, chlorine is placed first in order, since it seems to be required in greater amounts than any of the others. Cultures from which chlorine is withheld are designated M6 + (m7 − Cl).

Stock solutions of the four salts supplying the macronutrients were stored individually at molar concentrations. Of the micronutrients, iron was added separately as 0.002 *M* $FeSO_4$ adjusted with H_2SO_4 to pH 3.5. The iron solution was added twice weekly to each culture at the rate of two ml per litre. The six remaining micronutrients were kept in one solution, each at one thousand times the strength shown for the final culture. Thus, addition of one ml per liter of culture solution provided a single strength micronutrient supply.

At transplanting, to 4-liter pyrex culture vessels, solution salt concentrations were brought up to one-half strength. Additional one-half strength

levels were given at the end of one week, and single-strength levels at three weeks, five weeks, *etc.*, as required by crop species, seasonal vigor, or other need for additional mineral elements. All chemical compounds used to prepare the culture solutions, except H_2MoO_4 and H_2SO_4, were recrystallized twice. Our tap distilled water was passed through an activated charcoal filter to aid in removal of oxidized chlorine which might have been distilled from the chlorinated city water suppy. Following filtration it was distilled once more into an all-pyrex condensing and storage system. Plant stems were supported with fibrous non-absorbent cotton in the holes of the lids of the culture vessels. Recently, fibrous Dacron has been substituted for cotton because the latter is rather free of chlorides. Dacron wool may contribute a maximum

TABLE I

Macronutrient solution medium — M6 *				
Specific salts used		Concentration of individual elements		
Compounds	Single strength μ moles per liter	Elements	Single strength μg atoms per liter	ppm
KNO_3	6000	N **	16000	224
$Ca(NO_3)_2.4H_2O$	4000	K	6000	235
$NH_4H_2PO_4$	2000	Ca	4000	160
$MgSO_4.7H_2O$	1000	P	2000	62
		S	1000	32
		Mg	1000	24
Micronutrient solution medium — m7				
Specific salts used		Concentration of individual elements		
Compounds	Single strength μ moles per liter	Elements	Single strength μg atoms per liter	ppm
KCl	50	Cl	50	1.77
H_3BO_3	25	B	25	0.27
$MnSO_4.H_2O$	5.0	Mn	5.0	0.274
$FeSO_4.7H_2O$***	4.0	Fe***	4.0	0.22
$ZnSO_4.7H_2O$	2.0	Zn	2.0	0.131
$CuSO_4.5H_2O$	0.5	Cu	0.5	0.032
H_2MoO_4	0.1	Mo	0.1	0.0096

* Solution pH at single strength = 4.7.

** Total-N includes 14% NH_4-N and 86% NO_3-N which maintains pH near to 5. For control near pH 6 use:

1000 μ moles/liter $(NH_4)H_2PO_4$ plus 1000 μ moles/liter $(NH_4)_2HPO_4$.

With the latter selection of ammonium phosphate salts the total-P level remains constant, but the ratio of NH_4 to NO_3 changes from 14% to 21.4% and the total nitrogen in the culture is increased to 17,000 μg atoms per liter.

*** Iron is added separately, twice weekly (2 × weekly) at the 1S (single strength) level indicated. Stock $FeSO_4$ prepared as 0.002 *M* $FeSO_4$ in H_2SO_4 to pH 3.5 for solution stability in storage; take 2.0 ml stock per liter culture medium.

approximating 2 μg atoms of chlorine per culture. Non-absorbent cotton has been found to contain variable amounts of chloride. Cotton wool may contribute a maximum of approximately 20 μg atoms of chlorine per culture. When it is used to support seedlings in the cover of the culture solution, this chlorine source is reflected in better growth of plants. It is presumed that spray from aeration leaches chlorides contained in the cotton, into the culture solutions. All vessels and equipment were washed with nitric acid and rinsed with redistilled water.

Seeds were germinated in pyrex vessels in a $^1/_{80}$-strength M6 culture solution. Five plants were transplanted to each 4-liter pyrex culture vessel as soon after germination as their size and mechanical strength allowed. After one week, one of the five plants was removed, leaving the four most similar plants.

The macronutrient salts were recrystallized to reduce their contents of chlorine since recrystallization was found more effective than our former method of precipitating halides first with silver and subsequent removal of excess silver with hydrogen sulfide. Analyses of these salts for chlorine, showed that 2.5 μg atoms per 4-liter culture (at the single-strength level) was the degree of purification attained. The distilled water was reduced to 0.3 μg atoms of chlorine per liter. Since 10 to 20 liters of water were required by the plants over the six weeks growth period, 2 to 4 μg atoms of chlorine were supplied from the water. Gas scrubbing chains gave estimates of 3×10^{-6} μg atoms per liter of air used for aeration. Filtration of 45,000 liters of air from the atmosphere through dry Whatman No. 1 filter papers showed an average of 7×10^{-6} μg atoms per liter capturable in this way, but there was no way to ascertain how much of this might be made available to growing plants. Other tests with filter papers exposed on greenhouse tables, without air passing through them, showed the possibility of airborne particulate matter settling at the rate of 0.14 μg atoms per square inch per week. Variable amounts of chlorine were found between species of seeds. These are reported in Table II. The maximum chlorine contamination in the culture medium which could be accounted for from all except unrestricted airborne sources was 12μg atoms per culture. Subsequent experiments have been designed to reduce the extrinsic contamination.

All plants, except the tomato and alfalfa, were harvested seven weeks from

TABLE II

The chlorine content of seeds, μg atoms per seed			
Lettuce	0.01	Barley	1.0
Tomato	0.03	Alfalfa	0.04
Cabbage	0.02	Buckwheat	0.05
Carrot	0.16	Corn	2.7
Carrot (washed)	0.03 *	Beans	2.3
Sugar beet	0.33	Squash	0.04

* Carrot seeds were the only ones showing measurable losses of chlorine on washing with water.

transplanting. Alfalfa plants were cultivated for 14 weeks, during which time two cuttings were made before the final harvest.

Plant species used: The following commercial species were used: Compositae, *Lactuca sativa* var. capitata (New York Iceberg Lettuce); Solanaceae, *Lycopersicum esculentum* var. Marglobe (Tomato); Cruciferae, *Brassica oleracea* var. capitata (Copenhagen Market Cabbage); *Umbelliferae, Daucus carota* (Red Cored Chantenay Carrot); Chenopodiaceae, *Beta vulgaris* var. Crassa (Strain No. 22 U.S., Sugar Beet); Graminae, *Hordeum vulgare* var. atlas (Barley); *Zea mays* (Sweet Corn); Leguminosae, *Medicago sativa* (Alfalfa); Polygonaceae, *Fagopyrum esculentum* (Buckwheat); and Fabaceae, *Phaseolus vulgaris* (Dwarf Bush Bean).

Chlorine additions: Since the object of the experiment was to compare only the effect of chlorine in additions to culture solutions, no chlorine was added to the "minus chlorine" group, M6 + (m7 − Cl). At the time of transplanting, 100 μg atoms of chlorine per liter were added as ammonium chloride to the "plus chlorine" group, M6 + m7, making 3.5 ppm of chlorine in solution.

To preclude any chlorine deficiency in the "plus chlorine" control plants, on the fifth week one millimole per liter of potassium chloride was included with the periodic additions of nutrient salts. Thus, for the last two weeks the "plus chlorine" cultures had about 14 per cent more potassium in them than the "minus chlorine" cultures. Potassium chloride was selected because the cultures were already more than adequately supplied with potassium and it was felt that addition of chlorine through the medium of this salt would cause least disturbance to the experimental arrangement.

OBSERVATIONS AND RESULTS

Comparative growth and development

Marked differences in yields and chlorine contents were observed; these are summarized in Table III and Figure 1.

Lettuce: Chlorine-deficient lettuce plants first showed symptoms as wilting. Root growth was restricted, with the first evidence of unusual root development appearing as a many-branched habit of new lateral roots. These root laterals were stubby and developed club tips. Throughout the growth period the difference in rate of growth of roots was one of the most obvious features of the two chlorine levels. No destructive symptoms of mottling developed in the lettuce leaf until about four weeks after transplanting the seedlings. Because of the severe wilting during bright days, it was surprising that the leaves of the chlorine-deficient plants should have remained as free from secondary injuries as they did. Later,

TABLE III

Species	Plant part	Average yield g dry weight per culture		Chlorine in deficient plant material, μg atoms per g dry matter	Yield of chlorine-deficient plants, % of normal	Total chlorine per culture		Ratio of chlorine recovered to known
		No Cl added	Plus Cl			From known sources of contamination, μg atoms	Recovered from deficient plants at harvest, μg atoms	
Lettuce	Shoots	11.6	40.6	4.0	29	7.5	60.0	8.
	Roots	2.4	6.7	5.6				
Tomato	Leaf blades	4.9	13.4	6.5	36	12.0	66.0	5.
	Stems, petioles	4.1	11.7	4.7				
	Roots	1.6	4.8	9.4				
Cabbage	Shoots	26.6	66.8	1.5	42	7.5	66.6	8.
	Roots	4.1	7.1	5.9				
Carrot	Shoots	6.2	14.6	2.5	47	7.5	40.0	5.
	Roots	4.9	8.9	5.1				
Sugar beet	Leaf blades	10.9	23.6	1.7	49	7.5	60.0	8.
	Petioles	7.2	17.9	2.3				
	Roots	11.1	18.6	2.1				
Barley	Shoots	35.6	55.2	4.0	65	7.5	190	25.
	Roots	15.0	22.3	3.2				
Alfalfa 1st:	Leaf blades	5.8	12.4	3.4	70	10.0	173	17.
	Stems, petioles	6.3	11.6	2.4				
2nd:	Leaf blades	5.5	8.7	2.9				
	Stems, petioles	5.6	9.1	2.7				
3rd:	Leaf blades	8.4	10.0	3.4				
	Stems, petioles	14.1	18.4	2.3				
	Roots	18.1	20.9	2.4				
Buckwheat	Leaf blades	3.6	4.6	2.5	73	7.5	25.0	3.
	Stems	2.9	4.4	2.8				
	Roots	2.1	2.8	3.6				
Corn	Leaf blades	27.2	31.8	3.0	78	7.5	243	32.
	Stalks	76.8	97.0	1.4				
	Fruit	18.7	26.2	1.5				
	Roots	14.2	20.6	1.8				
Beans	Leaf blades	33.5	33.2	5.1	92	7.5	333	44.
	Stems	24.5	27.4	2.3				
	Fruit	25.2	30.6	2.8				
	Roots	14.8	15.6	2.3				
Squash	Leaf blades	22.6	21.3	2.9	102	7.5	203	27.
	Stems, petioles	44.1	41.8	1.2				
	Fruit	31.5	31.5	1.9				
	Roots	5.9	4.4	4.6				

Plate I. Lettuce cultures with deficient (left) and adequate (right) chlorine supply.

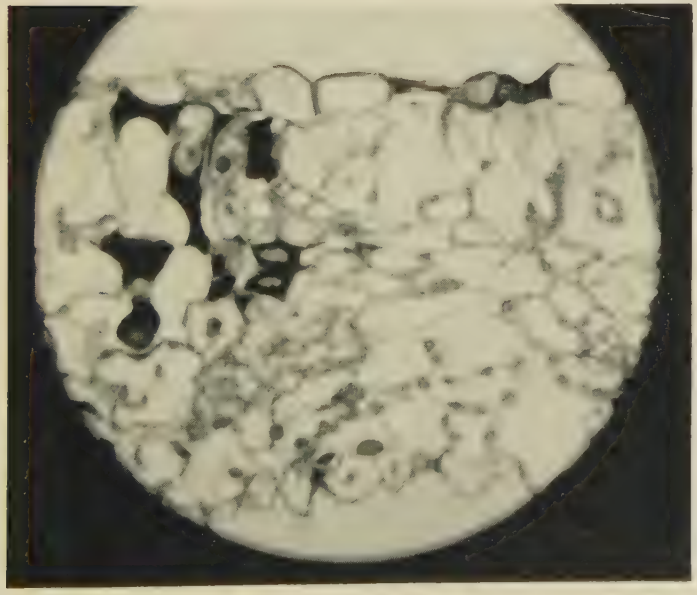

Plate II. Cytological disintegration in bronzing of tomato leaf from chlorine deficiency supply; the darkly stained areas in the tissue section are components of the bronzed areas in the leaf.

Plate III. Cabbage cultures with deficient (left) and adequate (right) chlorine supply.

Plate IV. Detail of wilting and cupping of cabbage under conditions of inadequate chlorine supply.

necrosis occurred on the more severely wilted leaves. Growth was restricted from the time wilting first appeared in the "minus chlorine" lettuce plants. The differential growth was cumulative. These differences in vigor are expressed best by Plate I which gives

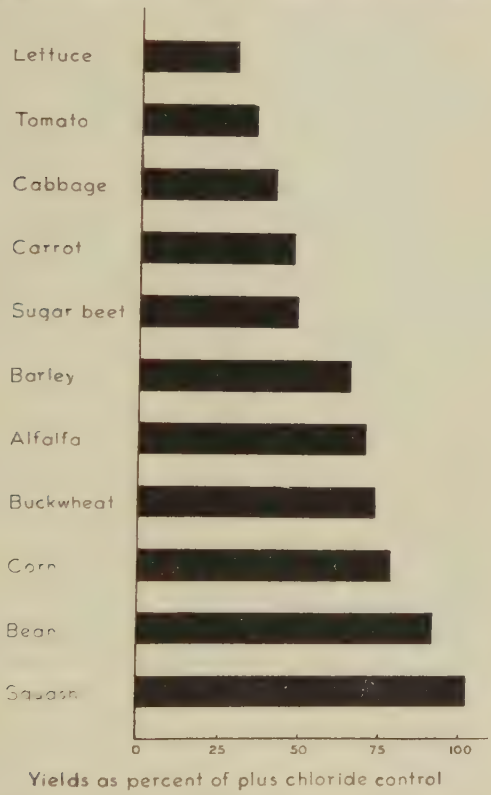

Fig. 1. Plant yields from chlorine-deficient cultures as per cent of those from adequately supplied chloride controls

a direct comparison of lettuce plants grown on cultures having no chlorine other than that supplied in the M6 + (m7 Cl) solutions and those which received M6 + m7 solutions which provided 100 μg atoms of chlorine per liter (3.5 ppm) at the time of transplanting. The three replicates within each treatment were so much alike that selection of any single culture was proper for photographic comparison.

The total dry weight yield of the "minus chlorine" lettuce plants was 30% of the "plus chlorine" plants. However, yield alone is an

inadequate expression of the real differences between the two treatments. Quality considerations would permit rating the latter plants as good. Their heads had begun to fill and were in every way indicative of an acceptable product by commercial standards. By contrast, the "minus chlorine" plants did not head and would be considered complete failures when invoking standards of market acceptance. There is no question that chlorine is required for the satisfactory growth and development of the lettuce plant.

Tomato: There is no need to review at length here the effects with the tomato plant since an account of the symptoms of chlorine deficiency is given in the earlier report [1]. Comparative root development between "plus" and "minus chlorine" cultures is shown in Figure 2. Again, as was noted previously for lettuce, the lateral roots of chlorine-deficient tomato plants were many branched and stubby, with club tips. This is in contrast with the usual fibrous type of root growth of the tomato plant.

In a special study, J. T. Woolley has partially characterized the chlorine-deficiency bronzing through histological observations. A photomicrograph of a tomato leaflet cross section through a bronzed area is reproduced as Plate II. The tissue thickness was 2 μ, the field of view about 130 μ. The tissue breaks down within individual cells. The destruction has no particular relationship to specific leaf structures, occurring in palisade, spongy tissue, or epidermis. It is sometimes adjacent to veins and at other times apparently includes xylem and phloem. The bronze-bodies take up safranin stain markedly (the basic stain used was fast green — not localized in bodies). The bodies are insoluble in water, xylol, ethanol, methanol, tertiary butanol, methyl cellosolve, weak (5%) HCl or weak (5%) NaOH. Seemingly, an entire cell breaks down into some form of proteinaceous coagulum which appears to fill some intercellular spaces between healthy cells. There is no evidence for a "secretion" from cells. This coagulum is amber in color when unstained and probably gives the bronze color to the leaf tissue affected. Further, chlorine-deficient and chlorine-sufficient leaves of the same age, size, and position had the same general histological structure at the time of onset of chlorine-deficiency symptoms in the "minus chlorine" leaves.

Fig. 2. Comparative lateral root development of tomatoes in inorganic solution cultures with (left) and without (right) chlorine additions.

Cabbage: As with the lettuce plants, there was marked restriction in the growth of cabbage cultured on the M6 + (m7 — Cl) solutions, best exemplified by the comparison of the two cultures shown in Plate III. Over-all growth and quality differences are as striking as with the lettuce plant. Cabbage plants were more variable between individuals than with lettuce. The culture with chlorine-deficient cabbage plants on the left of Plate III shows one of the plants less affected than the others. Even so, this particular plant was smaller than any single plant of the culture shown on the right which was supplied with 3.5 ppm chlorine at the beginning of the experiment. A curious point of comparison between cabbage and lettuce is the habit of root growth. With the "minus

chlorine" lettuce plants, inhibition of root growth was obvious very early in the growth period, but with cabbage no such visual difference was observed. Actually, the roots of the cabbage plants were longer in cultures not receiving added chlorine than in cultures which did. However, yield data given in Table III show that the weights of roots from the chlorine-deficient cabbages were 57% of those from the "plus chlorine" cultures. Like all other plants grown with limited supplies of chlorine, cabbage showed obvious water stress. Whenever a leaf was exposed to full sunlight, wilting took place at the tips and margins. The type of leaf wilting of cabbage can be seen in Plate IV. It appears that the wilted outer parts of the leaves do not expand as rapidly as the inner sections, which causes cupping of the cabbage leaves to take place. Sometimes the wilted leaf margins droop downward, leaving the cup shape inverted; on other occasions leaves wilt with margins turning upward in which event the cup shape is upright. Both types of cupping can be seen in Plate IV. Later in the growth period the most newly formed leaves of the chlorine-deficient cabbage plants became chlorotic. As with lettuce plants, cabbages inadequately supplied with chlorine were of such poor quality that they would not be considered acceptable from the point of view of quality. A second point of curious behaviour in cabbage was that the chlorine-deficient plants had no detectable cabbage odor whereas those receiving chlorine additions did. No reasons for this peculiarity are offered except to suggest that under conditions of water stress, transfer of gases from the interior of the leaf might have been lessened. Another possibility is that under conditions of severe growth restriction, unsaturated aldehydic constituents responsible for the characteristic cabbage odor are synthesized in smaller quantity.

Carrot: The carrot plant was restricted in growth of tops and roots as may be seen in Plate V. The root habit of the "minus chlorine" carrot plants tended toward many-branched laterals, a characteristic already noted for the "minus chlorine" lettuce or tomato roots. Carrots displayed the wilting symptom of chlorine deficiency less than any other species. However, about six weeks after transplanting a number of small leaflets turned brown and died. Storage-root development was greatly depressed on the

Plate V. Carrot cultures with deficient (left) and adequate (right) chlorine supply.

Plate VI. Sugar beet cultures with deficient (left) and adequate (right) chlorine supply.

Plate VII. Alfalfa cultures with deficient (left) and adequate (right) chlorine supply. Photograph taken just prior to first topping.

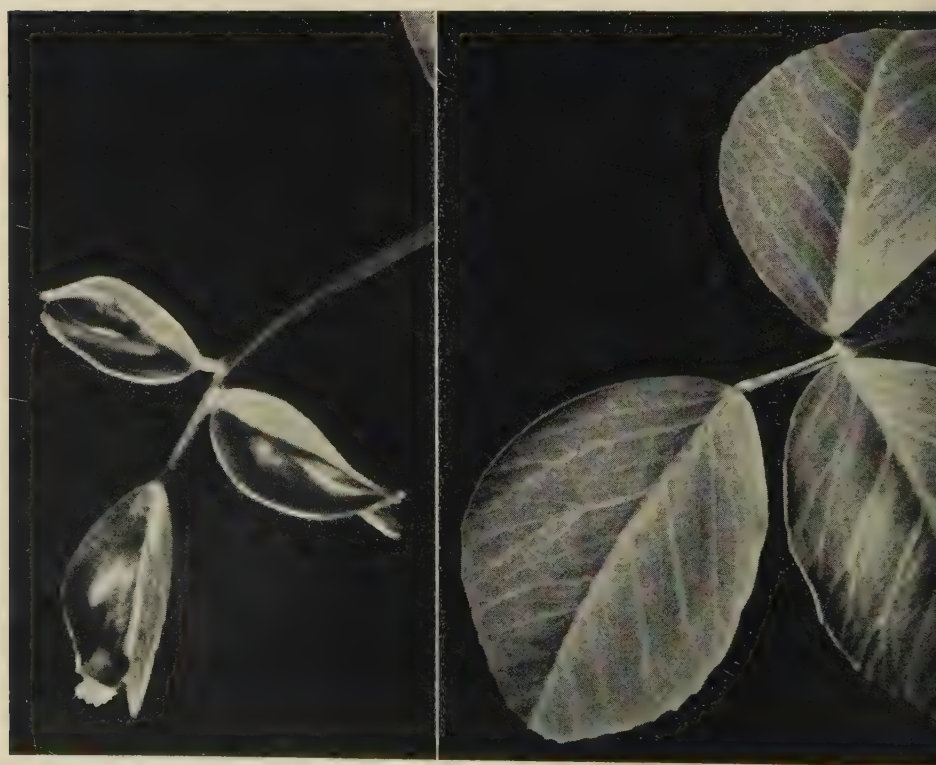

Plate VIII. Left: detail of alfalfa leaflets showing tip and lrregular laminar necrosi under conditions of inadequate chlorine supply.
Right (at the same scale): alfalfa leaflets under conditions of adequate chlorine supply

"minus chlorine" plants, but substantial storage roots developed on those having an initial concentration of 3.5 ppm chlorine in their cultures. Consequently, these chlorine-deficient carrot plants failed to meet the agricultural requirement of producing storage roots.

Sugar beet: From yield data the sugar beet was fifth in order of sensitivity to low chlorine, but from observations made at any time during growth, the sugar beet plants suffering from a limited supply of chlorine were most easily recognized. Since sugar beet plants tend to wilt more readily than plants of other species, the effects of chlorine depletion seem to be magnified when judged by the wilting symptom. The club-like root morphology with many-branched laterals of the "minus chlorine" sugar beet plants contrasted distinctly with the fibrous development of the "plus chlorine' roots, as may be seen in Plate VI. As with carrots, storage root development was repressed under limiting chlorine supply.

An extensive study of the chlorine nutrition of the sugar beet plant has been made by Ulrich and Ohki [6]. They present descriptions of manifestations of chlorine-deficiency symptoms in mature sugar beet plants and analytical data relating chlorine levels in plants and culture solutions to production of sugar.

Barley: The barley plant comes into the group showing exceptionally high amounts of chlorine acquired from extrinsic sources. Even so, during the seven weeks of growth, the yield of the sets of plants from the M6 + (m7 — Cl) cultures were depressed to 65% of that from cultures having intentional chlorine additions. The most distinctive symptom of the chlorine-deficiency disease in barley was a general chlorosis of newly emerging leaves. Besides being chlorotic, the leaves remained wrapped in tubular form much longer than normal barley leaves and were slower growing, smaller, and more fragile than comparable leaves of plants having received added chlorine. The former often became necrotic, similar to "wither-tip" in *Graminae* where Cu is limiting in supply.

Alfalfa: The alfalfa plants were clipped three times to simulate the usual method of harvesting. Also, they were allowed to grow beyond the seven-weeks period allotted to the other species. At the time of the first cutting the "minus chlorine" treatments

displayed prominent chlorine-deficiency symptoms as shown in the comparative photograph, Plate VII. It is of especial interest that the roots of these alfalfa plants did not seem to be affected adversely. Some alfalfa leaflets, though expanded before the intrinsic chlorine contaminants had become diluted low enough through plant growth to become limiting, appeared to retrogress, and decreased in leaf area perhaps by virtue of leaf thickening at the expense of lateral expansion. Plate VIII shows the contrast in size and shape of leaves of the same age which developed on alfalfa plants of the M6 + (m7 — Cl) and M6 + m7 culture solutions. These leaflets werep hotographed while on the plant with the same plate-to-lens setting. Consequently both photographs are in the same scale. Tips of the chlorine-deficient leaflets had split along the midrib and irregular chlorotic blotches appeared in the central portions.

Strangely, the second cutting of alfalfa showed less percentage yield depression of the "minus chlorine" plants than for the first cutting. The dry weight of the tops from the first cutting of these plants was 50% of the "plus chlorine" controls; and for the second cutting, 62%. At the third cutting, the yield from the M6 + (m7—Cl) culture group was 80% of that of the controls and the severity of symptoms had considerably diminished. At final harvest (14 weeks after transplanting) weights of the combined cuttings and roots of the "minus chlorine" group were 70% of the controls. The total chlorine recovered in the "minus chlorine" plants was 173 μg atoms per 4-liter culture — about 17 times more than could be accounted for from the chlorine known to have been in the nutrient salts and supplied by water during the growth period. For the alfalfa plant, it can be inferred that chlorine is necessary for its growth and development. Most curious, however, were the following: *a*) the healthy appearance of "minus chlorine" alfalfa roots at all times, and *b*) the large amounts of chlorine acquired by the plants above that which could be accounted for in seed, culture solutions, and added water.

Buckwheat: From the point of view of an experimental plant, buckwheat was the least satisfactory in the series. Under the experimental conditions, it proved to be slow growing and low yielding. At no time during the seven weeks did the M6 + (m7 — Cl) cultured buckwheat plants display symptoms of sufficient promi-

nence to classify them with assurance as being chlorine deficient. At the final harvest the yield of the "minus chlorine" plants was 72% of the "plus chlorine" plants. These yield differences were significant at the 5% level. Buckwheat, like all the other species of the experiment, acquired more chlorine than could be accounted for in the culture solution and added water.

Corn (maize): Corn plants showed excellent growth in these cultures, yielding considerably more dry weight of plant material than any other species within the series. The "minus chlorine" plants displayed the smaller yields, but with the exception of wilting, no leaf symptoms could be seen at any time during growth. Shortly after transplanting corn seedlings to the M6 + (m7 — Cl) culture solutions, they were noted to wilt during hot, bright periods of the day while those on M6 + m7 cultures did not. This species thus gave suggestions of chlorine-deficiency symptoms earlier than any of the others. Consequently, close observations were made for further expressions of abnormality. However, throughout the growth period no other signs of distress or nutritional disease were evident even though the wilting symptom persisted. Only younger leaves of the "minus chlorine" plants wilted and conditions of high light intensity were required to produce this symptom. During intervals of reduced light intensity they regained turgor. This difference in tendency to wilt between plants in M6 + (m7 — Cl) and M6 + m7 cultures, continued throughout the weeks, but otherwise they did not seem to be affected nor was it possible to judge whether or not differential yields could be expected. However, dry weights taken after harvest revealed that the "minus chlorine" plants were 78% of the "plus chlorine" controls, with the difference being significant at the 10% level.

Beans: The only significant difference noted with bean plants from the M6 + (m7 — Cl) and M6 + m7 cultures was found in their yields at the time of harvest. Bean plants cultured on these two solutions resembled each other seven weeks after transplanting. Comparison of plant yield data of Figure 1 clearly demonstrates the inherent differences in species effect under similar, inadequate chlorine environments. All of these plants were cultured on solutions from the same salt source and grew at the same time in the same

greenhouse. The been plants acquired very much more chlorine during their growth than the amount originally made available through culture solutions. The total chlorine recovered at harvest was over forty times the amount known to have come from salts, water, and seed. It is not known how much of the extrinsic chlorine having been captured was actually incorporated into the plant in a way to contribute to its physiological processes, but it is suggestive that a high proportion of the chlorine found by analysis was indeed incorporated into the plant in physiologically effective locations. The chlorine contents of 4.0 and 5.1 μg atoms per gram respectively for lettuce and bean leaves (Table III) show that the spreading habit of bean leaves did not give rise to much higher chlorine contamination. We can assume advisedly that the greater advantage in leaf spread captured a greater amount of chlorine, but that this added amount of chlorine resulted in correspondingly more plant growth so that the concentration of chlorine was increased very little. These experiments are to be repeated in a special section of our greenhouses which is being equipped with air-scrubbing devices.

The only symptom exhibited, which was distinctive as between the two treatments, was increased nastic movement of the leaves of the "minus chlorine" plants. For example, it was observed on several occasions that at sunset all the bean plants of the M6 + + (m7 — Cl) treatments could be recognized visibly by reason of their leaves being turned toward the sun, whereas their counterparts, having been supplied with chlorine, were distinctly less oriented. It is suggested that the general tendency of the "minus chlorine" plants to be under stress for water might have allowed greater freedom of leaf movement since the cells would be under less turgor.

Squash: The only suggestion of difference between squash plants with or without chlorine application was a chlorosis of the younger leaves of plants growing on the M6 + (m7 — Cl) cultures. These symptoms were rather transitory. Yields were not decreased by restricted chlorine supply. Actually, the average yield of the "minus chlorine" squash plants was 2% greater than those receiving added chlorine; although this difference was not significant at the 50% level.

DISCUSSION

Status of chlorine nutrition of crop plants

With the realization of the kinds of difficulties besetting experimental demonstrations of chlorine as an essential element for higher plants, it becomes understandable why the subject of chlorine in plant nutrition has remained uncertain for so many years. In retrospect, further strength is given to the suggestions of other investigators that chlorine is required for growth of higher plants. Of the work successfully demonstrating lower yields from "minus chlorine" plants cultured under controlled salt environments, several other species must be considered. Lipman [3] used buckwheat and peas; Eaton [2] tomato and cotton; and Raleigh [5] table beets. For each of these species, lowered plant yields resulted from attempts to exclude chlorine from the culture solutions. There have been many suggestions by agronomists and others that chlorine (usually added as KCl) may influence favorably the turgor of field crops. However, we do not feel that the latter experiments had been refined sufficiently to warrant more than the suggestions. Since chlorine-deficient plants have been produced and chemical analyses have been made of them, we are in position to deduce certain possibilities of economic implication.

Since the plant requirement for chlorine was found to be higher than for any of the other micronutrient elements, the reason for chlorine having escaped notice for so many years must have been its ubiquitous distribution in nature and many uses in industry. As a result, it has been an ever present source of contamination during the manufacture of chemicals needed for the compounding of culture solutions as well as in the growth environment of plants.

In the leaves of tomato plants suffering from acute chlorine deficiency, chlorine is present in the order of 1–3 microgram atoms (35–105 ppm) per gram dry weight of leaf tissue. By contrast, the micronutrient presently recognized as required in least amounts is molybdenum. Molybdenum-deficient tomato plants with equal visual stress will contain 0.001 of a microgram atom per gram (0.1 ppm). Thus, on an atomic-equivalent basis, the chlorine requirement is in the neighborhood of 1,000 times greater than that for molybdenum.

Chlorine needs of higher plants in relation to bromine [4 6] have

shown that bromine exercises a pronounced "sparing effect" for chlorine. Other evidence secured in our laboratories shows that fluorine does not, nor does iodine where supplied at lower levels (up to 2 μg atoms per culture). At levels approximating those effective in Cl "sparing action" with bromine (500 μg atoms per culture), there is the possibility that iodine may participate in chlorine functions, but toxicity symptoms from the former interfere with observations. Plants having very low amounts of chlorine cannot be brought up to full yield irrespective of bromine added [4] although moderate deficiencies can be corrected with bromine [6]. In nature, the amounts of bromine are so much less than chlorine, that it is doubtful whether bromine is at all significant in providing supplements to the chlorine available for plant use.

From a soil-fertility point of view it is estimated that plants require one pound of chlorine for each 10,000 pounds of dry matter produced. Large crops would therefore need 2 or more pounds of chlorine per acre. Chlorine, unlike other nutrient elements contained in native rocks, is not fixed by soil colloids. It may be assumed that shortages of chlorine would have been observed long ago unless some process has been replacing regularly the chlorine leached away in drainage waters. A source of continued supply may be found in the chlorine content of rain waters. Many analyses of annual chlorine acquisitions from the atmosphere have been made in many parts of the world. Lower recorded values are in the order of 10 pounds of chlorine per acre per annum with many coastal stations reporting deposition of hundreds of pounds of chlorine per acre.

SUMMARY

Recognition of chlorine as a plant micronutrient has been extended to include ten species. Acute chlorine deficiencies or decreased yields were produced with lettuce, tomato, cabbage, carrot, sugar beet, barley, alfalfa, buckwheat, corn, and beans. Squash plants showed neither loss in yield nor other deficiency symptoms when cultured at the same time and under the same conditions as the aforementioned species. All plants acquired more chlorine during their growth than can be accounted for from seeds, inorganic salts, or water used in the experiments. Plant species least susceptible to injury when cultured upon low chlorine salt solutions were also the ones most capable of acquiring extrinsic chlorine. Of the species studied, lettuce was the most senstitive to "minus chlorine" culture solutions and squash, the least sensi-

tive. However, the concentration of chlorine in all of the species cultured under limited chlorine supply was not greatly different. It is inferred that plants such as corn, beans, and squash survived the "minus chlorine" cultures by reason of greater accretion of extrinsic chlorine from the atmosphere. The form of the atmospherically borne chlorine is not known.

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ASSIMILATION OF BERBERINE AND CHELIDOXANTHINE BY BACTERIA *

by P. H. H. GRAY and R. A. LACHANCE **

INTRODUCTION

It has recently been found in this laboratory [2 5] that many bacterial species can grow well in media prepared with aqueous extracts of *Chelidonium majus* L. (celandine or swallowwort) and *Berberis vulgaris* L. (barberry). These plants contain the yellow fluorescent alkaloids chelidoxanthine and berberine; the latter alkaloid is also associated with the blue fluorescent alkaloid hydrastine in *Hydrastis canadensis* L. (golden seal). Berberine is found also in roots of *Mahonia aquifolium* Nitt. (Mahonia) and in several other plants. Wehmer [8] states that berberine is found in the roots of *Berberis vulgaris* in the concentration of 1.3%, presumably in the dried root bark ("Wurzelrinde"); this is a much higher concentration than that reported for chelidoxanthine in fresh *Chelidonium majus*, 0.01%. He also identified chelidoxanthine with berberine.

EXPERIMENTAL

Extracts were prepared by boiling fresh or dried plant materials in distilled water. In the first test the foliage of *Berberis* of *Chelidonium*, and the roots of *Mahonia* were used. The filtered extracts yielded, on the paper, a bright yellow fluorescence in filtered ultra-

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** Professor and Chairman, Department of Agricultural Bacteriology, and Graduate Student, Faculty of Graduate Studies and Research, McGill University, Montreal, Que., respectively. The work formed part of that done by the junior author in partial fulfillment of the requirements for the Ph. D. degree of McGill University.

violet light; the *Chelidonium* extracts were lemon-yellow in colour, and those of the *Berberis* and *Mahonia* had a greenish * hue. When extracts were added to beef—peptone agar, the growth of bacteria on the surface of slopes and plates exhibited a yellow fluorescence in ultra-violet light †; on the agar without the extracts they were not yellow, thus suggesting that the bacteria while growing had assimilated the fluorescent compounds of the plants. Extracts of various other plants, containing other alkaloids, both with beef—peptone agar (Bacto Nutrient Agar) and with plain agar (Bacto Agar) **, were also made and inoculated with various bacteria. After incubation at 37° for 48 h the bacteria that grew developed the yellow fluorescence only on the agars made with extracts from the plants containing chelidoxanthine and berberine. Other species of plants containing berberine were not available for study. The results of preliminary tests are shown in Table I; since all of the bacteria grew on Nutrient Agar, only the results from the Bacto Agar are shown.

TABLE I

Growth of bacteria on Bacto Agar containing extracts of *Berberis*, *Chelidonium* and *Mahonia*. (+ indicated growth and fluorescence).

Bacteria		*Berberis*	*Chelidonium*	*Mahonia*
M.C.C. No. §	Name			
50	*M. pyogenes aureus* H	+	+	+
40	*M. pyogenes aureus* McG.	+	+	+
17	*M. cereus*	0	0	0
41	*Sarcina* sp.	0	0	0
38	*Phytomonas phaseoli*	0	+	0
37	*Phytomonas tumefaciens*	+	+	+
33	*Proteus vulgaris*	+	+	+
36	*Pseudomonas pictorum*	0	+	0
34	*Ps. aeruginosa*	+	+	+
32	*Ps. syncyanea*	+	+	+

§ The M.C.C. No. is that applied to the culture in the Macdonald College Collection of Pure Cultures.

* This might be caused by blue fluorescence from hydrastine in *Berberis*; blue fluorescence may also be caused by amino compounds.

† The Hanovia ultra-violet lamp, 3660 A (provided by Fisher Scientific Co. Ltd., Montreal, Canada), and the Technico lamp of A. Gallenkamp, London, England, were used for assessing fluorescence colours.

** Bacto Nutrient Agar and Bacto Agar are names applied by the Difco Laboratories Inc., Detroit, Michigan, U.S.A. to beef-extract peptone agar and agar without added nutrients, respectively.

The non-fluorescent bacteria that grew on *Chelidonium* extract agars were bright yellow under ultra-violet light; those on *Berberis* and *Mahonia*, yellow-green. The yellow in the fluorescent species of *Pseudomonas* was masked by water-soluble fluorescent compounds in the medium (see also Table III). *Phytomonas phaseoli* and *Pseudomonas pictorum* were sensitive to the *Berberis* and *Mahonia* extracts, whereas one of the species of Micrococcaceae was sensitive also to the *Chelidonium*. The extracts alone, in Bacto Agar, provided enough food for growth of the non-sensitive cultures.

TABLE II

Showing the highest concentrations of *Chelidonium* and *Berberis* extracts at which bacteria grew on Bacto Agar and Nutrient Agar.

(1)	(2)	(3)	(4)	(5)	(6)
M.C.C. collection		*Chelidonium* in		*Berberis* in	
No.	Name	Bacto	Nutrient	Bacto	Nutrient
		%	%	%	%
45	*B. megatherium*	14	16	2	2 (s)*
7	*B. ruminatus*	14	16	2	3 (s)
16	*C. xerosis*	16	16	2	2 (s)
28	*M. phlei* M	4	7	1	1 (s)
47	*Pro. globerulum* M	3	2	0	1 (s)
53	*Pro. erythropolis*	8	8	3	4
—	*A. globiforme* (201–425)	1	2	0	1 (s)
—	*A. globiforme* (247) *	1	1	1	2 (s)
50	*M. pyogenes aureus* *	16	16	4	5
10	*Bact. iophagum*	3	5	8	8
2	*A. aerogenes*	16	16	8	8
12	*C. biazotea*	1	1	0	1 (s)
22	*E. coli*	16	16	8	8
33	*P. vulgaris*	16	16	4	8
34	*Ps. aeruginosa*	16	16	8	8
32	*Ps. syncyaneum*	16	16	8	8
36	*Ps. pictorum*	2	5	1	1 (s)
42	*Ps. desmolyticum*	16	16	8	8
38	*Ph. phaseoli*	3	4	1	1 (s)
37	*Ph. tumefaciens*	6	8	5	5
70	*S. marcescens* W.	16	16	8	8

* *Note*: *A. globijorme* 247 resembles species 201–425, received from the Division of Bacteriology, Department of Agriculture, Ottawa, except that it has flagella. *M. pyogenes cereus* and the *Sarcina* species did not grow except on Nutrient Agar without either extract. Those species marked (s) in column (6) are regarded as especially sensitive to berberine (see below under Table IV). Failure of Nos. 36 and 38 to grow in the former test may have been due to the ages of the *Berberis* and *Mahonia* plants.

Concentrations of extracts

The effects of different concentrations of the extracts were then observed after cultures of bacteria had grown on *Chelidonium* extract agar of 1 to 16 per cent extract, and on *Berberis* root extract agar of 1 to 8 per cent, with both Bacto Agar and Nutrient Agar. The results from 22 cultures, after 48 hours at 37°, are given in Table II.

The figures in columns 3 to 6 are those of the highest concentrations at which the bacteria grew and developed fluorescence.

Fluorescence colours

Different species produced varied fluorescence colours in ultra-violet light, depending upon the concentrations of the extracts. Most of the cultures on *Chelidonium* extract in Bacto Agar had a yellow or brown fluorescence at the lower concentrations and brown at the higher; on the extract with Nutrient Agar all of the cultures were yellow at the lower concentrations. A few of the cultures developed different fluorescence colours: such differences are given in Table III.

TABLE III

Fluorescence colours of certain species of bacteria growing on *Chelidonium* extract agars

Culture	Bacto Agar		Nutrient Agar	
	Concentrations	Colours	Concentrations	Colours
B. megatherium	all	blue-green	all	blue-green
B. ruminatus *	all	blue-green	all	blue-green
M. pyogenes aureus	1–3	lemon-yellow	1–8	lemon-yellow
	4–16	dark-yellow	9–16	yellow
Ps. desmolyticum	1–2	blue	1–7	yellow, blue edge
	3–16	brown, blue edge	8–16	yellow-green
Ps. aeruginosa	low	blue	low	blue, yellow edge
	high	green	high	green
Ps. syncyanea	low	blue	low	blue, yellow edge
	high	green	high	green

* The culture has since split into two strains, one of which is yellow and one blue green on *Chelidonium* extract agar, in ultra-violet light.

The colours of cell material transferred to filter paper were of some hue of yellow in ultra-violet light, at all concentrations of *Chelidonium*; cells washed after being centrifuged in distilled water were also yellow. The cultures on the *Berberis* agar were similar to

those on the *Chelidonium*, except that *B. megatherium* and *B. ruminatus* were lemon-yellow at all concentrations, and *Ps. desmolyticum* was blue on the Bacto Agar but yellow-green, with the edges of the growth blue, on the Nutrient Agar, at the lower concentrations. Some of the bacterial cultures thus differed in appearance according to the nature and concentrations of nutrients.

The production of blue-green fluorescence of the two species of *Bacillus* was further investigated by cultivating also six other species of this genus on extract agars in plate cultures. The cultures tested were No. 3, *B. cereus*, No. 6, *B. mesentericus*, No. 8, *B. subtilis* 85, No. 20, *B. subtilis*, No. 46, *B. mycoides*, and No. 78 *B. mycoides*, in addition to Nos. 7 and 45. Only the last two, namely, *B. ruminatus* and *B. megatherium*, had the blue-green fluorescence on *Chelidonium* agar. All of the cultures had a yellow fluorescence on *Berberis* agar.

Inhibition of growth by berberine

Other workers have shown [1 3 6] that low concentrations of berberine hydrochloride are toxic to certain bacteria and fungi in cultures. The apparently more repressive effect of the *Berberis* extract may have been due to the fact that berberine is more highly concentrated in the *Berberis* plant than the alkaloids (chelidonine, chelidoxanthine) are in *Chelidonium*. It will be noted, in Table II, that those cultures which were sensitive to the lower concentrations of *Chelidonium*, viz. Nos. 28, 47, *A.globiforme* (201) and (247), 10, 12, 36 and 38 were also the most sensitive to *Berberis*; the presence of added nutrients, in Nutrient Agar, did not much reduce that sensitivity.

These tests were continued, but with various concentrations of pure berberine hydrochloride *. Thirty-six cultures were inoculated onto Nutrient Agar containing 19 increasing concentrations of berberine hydrochloride, from 0.0025 to 2.0 per cent, and incubated at 37°. The results are shown in Table IV. All of the bacteria grew on Nutrient Agar without the alkaloid.

Those cultures which failed to grow at 0.1% and less may be considered as especially sensitive to the alkaloid. It will be noted that 9 of the 10 cultures reported previously (see Table II) as sensitive to the extract of *Berberis* were also sensitive to the alkaloid itself; the exception was *A. globiforme* 201–425 which was relatively

* From Brickman & Company, Montreal, Que., Canada.

TABLE IV

Bacteriostatic concentrations of berberine hydrochloride in Nutrient Agar			
Culture	Lowest bacteriostatic concentration %	Culture	Lowest bacteriostatic concentration %
* *B. megatherium*	0.03	*Bact. iophagum*	Above 2.00
* *B. ruminatus*	0.03	*A. aerogenes*	Above 2.00
B. cereus	0.20	* *C. biazotea*	0.01
(*) *B. subtilis* 85	0.02	*E. coli*	Above 2.00
(*) *B. subtilis*	0.02	*P. vulgaris*	1.00
(*) *B. mycoides*	0.06	*Ps. aeruginosa*	Above 2.00
* *C. xerosis*	0.05	*Ps. syncyanea*	Above 2.00
* *M. phlei* M	0.005	*Ps. indoloxidans*	1.00
(*) *M. stercoris* S.	0.02	* *Ps. pictorum*	0.10
* *Pro. globerulum*	0.02	*Ps. desmolyticum*	Above 2.00
A. globiforme 201	0.70	* *Ph. phaseoli*	Below 0.0025
* *A. globiforme* 247	0.03	*Ph. tumefaciens*	0.2
* *M. pyogenes aureus* H	0.03	*S. marcescens* W	Above 2.00

* Denotes species regarded as especially sensitive to berberine hydrochloride; .04% is approximately .001*M*. See note under Table II, and the text relating to this Table.

(*) Denotes sensitive species not used in the previous test. Several other Gram-negative cultures grew at the highest concentration tested (2%).

resistant to the pure alkaloid in that it grew at the 0.7 per cent concentration. *M. pyogenes cereus* and the *Sarcina* species did not grow at the lowest concentration. Most of the Gram-positive bacteria were sensitive; only three of the Gram-negative bacteria were affected by low concentrations: *C. biazotea*, a cellulose-decomposing species, *Ps. pictorum*, a phenol-decomposing species, and *Ph. phaseoli*, a plant pathogen.

The aqueous solution of berberine hydrochloride, when dried on filter paper, was brilliant yellow in ultra-violet light; the bacteria that had shown that colour after growing on *Berberis* extract agars were the same colour on the media containing the berberine hydrochloride.

Chelidonium *extract a complete food source*

As shown above, several of the bacteria grew in the sterilized extracts of the berberiniferous plants, though they grew better and developed a more intense fluorescence when provided with added nutrients. The two species *Pseudomonas aeruginosa* (34) and *Ps. syncyanea* (32) differed from the other species tested in that they pro-

duced gas when growing in *Chelidonium* (4%) extract agar. The diphenylamine test showed that the extract contained nitrate nitrogen *. The gas was found to be nitrogen; there was no detectable carbon dioxide or combustible gas in cultures grown in fermentation tubes. Both species produced larger volumes of nitrogen after growth in the 4% extract to which sodium nitrate 0.1% had been added. Both species produced the same volumes of gas in the extract with added glucose as they did in the extract alone.

Berberine not a source of carbon or nitrogen

Berberine hydrochloride alone in mineral salts agar medium did not support any growth. This was tested by inoculating eight bacterial species onto agar media composed of K_2HPO_4 0.1%; $MgSO_4.7H_2O$ 0.02%; NaCl 0.01%; $CaCl_2$ 0.01%; $FeCl_3.6H_2O$ 0.002%, and agar 1.5%, (Medium B1), to which the following were added to make three separate media:

glucose, 0.1% (Medium B2)
glucose, berberine hydrochloride (Medium B3)
berberine hydrochloride (Medium B4).

The pH of the media was adjusted to 6.8. Plate cultures were incubated at room temperature for 7 days.

Three of the bacteria grew, though but poorly, on Medium B1, and none on the other media. Ten species, including two of those three (Nos. 70 and 108) grew on the same glucose salts agar (Medium B1) provided with various sources of mineral nitrogen, and all grew and exhibited the lemon-yellow fluorescence on the same media containing berberine hydrochloride. The alkaloid was not, therefore, a source of either carbon or nitrogen in these media.

Fluorescence with amino acids

Since the fluorescence of the cells in the mass was more intense in or on extract media prepared with peptone, several bacterial species were inoculated onto mineral salts agar, with or without berberine hydrochloride 0.0025%, to which different single or variously grouped amino acids were added, and with glucose 0.1% as an additional source of carbon. The amino acids, both singly and grouped, provided 14 mg of nitrogen per 100 ml of medium. Twenty-three amino acid solutions were prepared as sterile stock solutions in

* This is believed to be the first evidence of free nitrate nitrogen in this plant species.

distilled water, so that 1 ml of each stock solution contained 1,4 mg of nitrogen. The pH of each medium was adjusted to 6.8.

Twenty-three media contained each a single amino acid; fifteen media contained each a different mixture of them: in seven of the fifteen the amino acids were grouped by "formula relationship", and in eight each related group was omitted in succession from the complete medium containing the 23 amino acids [7].

Twenty bacterial species were inoculated onto the sloped agars and examined after 48 h and 5 days. The fluorescence colours were assessed by transferring loopful scrapings of bacterial growth (paste) to filter paper. Certain pigmented bacteria, which have not been included in this report, had various colours. The results for 10 species are shown in Tables V and VI. The colours were assessed only approximately: Thus,'yellow' (Y) may indicate 'bright lemon yellow' or 'daffodil yellow', and degrees of intensity have not been compared.

The 'yellow green' (YG) and 'green yellow' (GY) ranges may indicate that the assimilation of some components of the medium, upon which, without the alkaloid, the colour of the cells was cerulean blue, interfered with the full yellow colour. The amount of cell growth might also have caused some variation in the amount of alkaloid assimilated *. The three colour groups Y, YG and GY, have been accepted as indicating a maximum intake of the alkaloid. The colour groups YB, BY, BW and B indicate either the development of the blue fluorescence from progressively greater intakes of components of the basic medium or interference by the development of fluorescence (fluorescein or pyocyanin) from such components, mainly in the cultures of the *Pseudomonas* species.

A two-way summation of the numbers of cultures in the media with separate amino acids (Table V) will show that 170 of 228 cultures were placed in the Y, YG and GY colour ranges; of the other 58, 57 were of species of *Pseudomonas* †. It is interesting

* The term "assimilated" has been used as a lexicological variant for the term "taken in", and its use here does not imply that evidence has been presented that the bacteria did more than "accumulate" the berberine hydrochloride. There is evidence, submitted below, that the berberine is held within the cell, and not merely on the surface, though held loosely; it appears also to be held in a form (?unchanged) assimilable by growing bacterial cells.

† The greatest intensity of fluorescence was observed in cultures growing with aspartic acid and glutamic acid, both separately and combined; the cells were also yellow in daylight.

TABLE V

Fluorescence colours developed by bacteria in media containing separate amino acids, with berberine hydrochloride 0.0025%.

Symbols: Y = Yellow; YG = Yellow tinged with green; GY = Greenish yellow; YB = Yellow tinged with blue; BY = Blue masking yellow; BW = Blue with lighter facies; B = Blue (cerulean); G = Dull green

Group No.	1	1	1	1	1	2	2	2	3	3	3	4	4	5	5	5	5	6	6	6	7	7	7
Bacterial species	glycine	alanine	*nor*-leucine	serine	threonine	cysteine	methionine	taurine	*iso*-leucine	leucine	valine	aspartic acid	glutamic acid	arginine	asparagine	lysine	ornithine	histidine	OH-*l*-proline	proline	phenyl-alanine	tryptophane	tyrosine
B. ruminatus	YG	Y	Y	Y	GY	Y	Y	Y	Y	Y	GY	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
B. megaterium	Y	Y	YG	GY	Y	YG	Y	Y	YG	GY	Y	Y	Y	Y	Y	Y	Y	Y	GY	Y	Y	Y	Y
P. erythropolis	YG	GY	YG	Y	Y	Y	Y	Y	Y	Y	GY	GY	GY	GY	GY	GY	Y	Y	GY	GY	GY	Y	YG
B. (A.) globiforme	YG	GY	Y	Y	—	Y	Y	Y	Y	Y	Y	GY	Y	Y	YG	Y	Y	Y	GY	GY	Y	Y	Y
B. (A.) aerogenes	YG	YG	—	Y	Y	Y	Y	Y	Y	Y	Y	GY	Y	Y	YG	YB	Y	GY	Y	YG	Y	GY	Y
B. (S.) marcescens W	Y	YG	Y	Y	Y	Y	Y	Y	Y	Y	Y	GY	Y	Y	YG	Y	Y	GY	Y	Y	Y	G	Y
Ph. tumefaciens	Y	YG	Y	Y	Y	Y	Y	Y	YG	B	YG	YG	YG	Y	Y	Y	Y	Y	GY	YG	Y	Y	Y
Ps. aeruginosa	B	BY	G	B	B	G	Y	Y	YB	YB	B	YG	G	B	BY	B	B	BW	B	B	B	G	G
Ps. desmolyticum	B	BW	B	Y	B	Y	Y	Y	Y	Y	B	YG	Y	BY	B	BY	B	B	B	BW	BY	G	BW
Ps. syncyanea	B	BY	G	B	B	G	Y	Y	YB	Y	B	YG	G	B	BW	B	B	BW	B	B	B	B	G

TABLE VI

Fluorescence colours developed by bacteria in media containing grouped amino acids, with berberine hydrochloride 0.0025%.
(For explanation of symbol letters, see Table V).

Medium No.	1	Separate group media, 2–14							Medium No. 1, successive groups omitted, 3–15						
Bacterial species	All (23) amino acids	mono-amino carboxylic	SH—	CH_3—CH—CH_3	mono-amino di carboxylic	di-amino carboxylic	heterocyclic	aromatic	omitting gp. 2	omitting gp. 4	omitting gp. 6	omitting gp. 8	omitting gp. 10	omitting gp. 12	omitting gp. 14
B. ruminatus	GY	Y	Y	YG	Y	YG	YG	YG	YG	Y	YG	Y	YG	YG	Y
B. megaterium	YG	Y	YG	YG	Y	YG	YG	YG	YG	Y	YG	YG	YG	YG	Y
P. erythropolis	YG	YG	YG	GY	GY	GY	GY	Y	YG	YG	YG	GY	YG	YG	YG
B. (A.) globiforme	YG	Y	Y	YG	YG	YG	GY	BW	Y	Y	YG	Y	YG	YG	YG
B. (A.) aerogenes	YG	Y	Y	Y	YG	Y	YG	Y	YG	Y	YG	YG	YG	GY	YG
B. (S.) marcescens	YG	Y	Y	Y	YG	Y	YG	Y	YG	Y	YG	YG	YG	GY	YG
Ph. tumefaciens	Y	YG	Y	—	Y	Y	YG	GY	Y	G	GY	Y	YG	YG	G
Ps. aeruginosa	BY	B	Y	BW	YB	B	YB	G	BW	BY	G	B	BY	BY	B
Ps. desmolyticum	BY	B	Y	BW	YB	Y	YB	BW	BY	BY	B	B	B	YB	B
Ps. syncyanea	BY	BW	Y	BW	YB	B	YB	B	BW	BY	G	B	BY	B	B

to note that all of the species on the B-HCl media containing the three sulphuretted amino acids were yellow, except *Ps. aeruginosa* and *Ps. syncyanea* on the cysteine medium. These two species were dull green (G) on *nor*-leucine, cysteine, glutamic acid, and tyrosine.

The results from the media containing the grouped amino acids are shown in Table VI. They confirm in part those obtained with the single amino acids. 105 of 148 cultures showed a maximum intake of the alkaloid; all but 3 in the other colour ranges were species of *Pseudomonas*. The grouped sulphuretted amino acids (Medium No. 4 in Table VI) also appeared to stimulate a maximum intake of the alkaloid, without exceptions among the 10 species. When group 4 was omitted (Medium No. 5) the cultures of *Phytomonas tumefaciens* and the three species of *Pseudomonas* developed the blue fluorescence, which masked their assimilation of the alkaloid. None of the other species appears to have been dependent upon any one group or combination of groups of amino acids, though the cells of all species were the more intensely yellow on the media containing aspartic acid and glutamic acid, Medium No. 8.

Re-assimilation of berberine from bacterial protoplasm

Evidence that the alkaloid was held within the cells was suggested by the results of an experiment in which washed cells of *B. ruminatus* were used to provide a source of organic nitrogen to other bacterial cultures. The cells were washed from surface colonies on Nutrient Agar and on Nutrient Agar containing the alkaloid, and then centrifuged from distilled water. The yellow fluorescence from the B.HCl medium was retained in the centrifuged cells, and very little in the wash-water. The bacterial pastes were then dispersed in water and the cells broken by glass beads, by means of a Mag-Mix * stirring apparatus. These bacterial suspensions were used to supply nitrogen in four media.

The following media were prepared, all with glucose 0.1% and agar 1.5%, and adjusted to pH 6.8 before sterilization.

I. Cells from Nutrient Agar.
   a. Cells only.
   b. Cells and peptone 0.1%.

* A magnetic stirring apparatus supplied by Fisher Scientific Company, Ltd., Montreal, Que., Canada.

II. Cells from Nutrient Agar containing B.HCl.

a. Cells only.

b. Cells and peptone 0.1%.

Plates of the above media were inoculated on the surface with 4 species of bacteria. After 2 days at 37° all of them had grown on the four media, and had developed yellow or yellow-green ultra-violet fluorescence on media II*a* and II*b*. The growth was more abundant and the fluorescence more intense on the media containing peptone. Clear zones, indicating proteolysis of the bacterial cell nitrogen, were observed around the colonies of three species (*B. ruminatus* [7], *B. megatherium* [45] and an unidentified species isolated from soil attached to the roots of *Chelidonium majus* and tentatively named *Bacterium unum* G. & Lach.).

SUMMARY

Several species of bacteria are able to assimilate the alkaloid berberine while growing in extracts of *Berberis vulgaris*, *Chelidonium majus*, and *Mahonia aquifolium*. They can also assimilate the alkaloid in nutrient media containing berberine hydrochloride in concentrations up to and including 2.0 per cent, but only if provided with suitable sources of carbon and nitrogen. Some bacteria, especially Gram-positive species, were found to be sensitive to relatively low concentrations of the alkaloid. The alkaloid appeared to be assimilated during the early stages of growth, and to be held in the protoplasm, for surface growth on agars and the washed cells had the bright yellow fluorescence of berberine, in ultra violet light. In mineral salts media with glucose and with amino acids as sources of nitrogen the intake of the alkaloid appeared to be stimulated by aspartic acid, glutamic acid, cysteine, methionine and taurine. Bacterial cells washed from colonies on agar containing the alkaloid served as a source of nitrogen to other bacteria, which extracted and assimilated the berberine from the washed cells.

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SOME PROBLEMS ASSOCIATED WITH DETERMINING IRON IN PLANTS

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It is well known that the total iron content of leaves determined by chemical methods seldom reflects the true iron status of plants. In instances of lime-induced chlorosis in fruit trees (Wallace [17], Bennett [1]) or metal-induced iron deficiency in plants (Nicholas and Thomas [11]), the iron content of chlorotic leaves is often reported to be similar to or even higher than that of green leaves.

A number of workers have shown that iron determined by leaf analysis is considerably less after washing leaves of fruit trees either in water containing a detergent or in dilute hydrochloric acid (Jacobson [4], Smith, Reuther and Specht [15], Mason [6]).

This paper amplifies a preliminary report (Nicholas, Lloyd-Jones and Fisher [12]) which showed that iron is present as a serious contaminant on leaf surfaces of tomato grown in a glasshouse and that it may be removed completely by washing the plants in water containing a detergent without loss of the trace metal from within the tissues. The investigation also showed no loss of iron during the acid digestion of various types of dried plant material specially chosen to give a wide range of silica contents.

EXPERIMENTAL

Soil culture

Tomato *var.* Market King was grown in a John Innes compost, (1.8 kg soil) in 6″ clay pots. All plants were grown in a glasshouse and some were put in dustproof chambers consisting of wooden frames covered with cellophane. In all experiments the treatments were randomised within blocks and replicated ten times. Iron-deficient plants were produced by adding heavy metals to the soil, *e.g.* Co or Ni sulphates.

Solution culture

Tomato seeds germinated on damp glass wool were transferred to 1-litre Erlenmeyer flasks containing culture solutions (Rediske and Biddulph [13]). One seedling was supported on a polythene platform which fitted into the neck of each flask. The culture solutions were maintained at a constant level and were aerated with air that had been passed through glass wool filters. The culture solution was freed from iron by the quinoline procedure (Nicholas [10]) after which 1 mg of carrier iron labelled with radioactive iron (Fe^{59}), both forms present as ferric chloride in HCl, was added together with an equivalent amount of disodium ethylene diamine tetra acetic acid. The pH of the culture solution was adjusted to 6 with sodium hydroxide which had been purified by ion exchange.

Preparation of plants for analysis

The tomato plants were cut at approximately 4″ above soil level when 9 weeks from seed, and the tops were immersed and agitated in 0.3 per cent "teepol" (sodium higher alkyl sulphate) in distilled water. Five seconds were allowed for drainage between immersions in successive solutions; the latter were renewed after three plants had been washed. The times of washing in these solutions were varied between 5 and 30 sec. The plants were dried in 400-ml pyrex beakers at 120°C for 1 h, and subsequently at 98°C for a further 12 h in an aerated oven.

Chemical methods

The plant material was digested in redistilled nitric acid and A.R. perchloric acid (Nicholas [8]). Wash solutions were evaporated nearly to dryness, then a nitric—perchloric acid mixture was added and digestion continued as for plant material. The fume cupboard used was lined with glass and the hotplate was made of aluminium, to avoid iron contamination. Iron was determined by α,α-dipyridyl (Moss and Mellon [7]), manganese by periodate (Richards [14]), and by tetrabase (Nicholas and Fisher [9]), potassium by sodium cobaltinitrite (Brown, Robinson and Browning [2]) and phosphate by vanadomolybdate (Hanson [3]).

Radioassay technique

Radioassay of Fe^{59} was made by solution counting, either in an EKCO scintillation counter or in an M_6 type G–M tube (Veal [16]). Isotopic decay and minor scaler fluctuations were allowed for by counting an aliquot of the solution used for the treatments after every three or four experimental solutions. The radioactive and carrier iron was added to the dried plant material in the recovery experiments as ferric chloride in dilute hydrochloric acid, and to the culture solutions as the chelate compound with ethylene diamine tetra acetic acid (Fe-EDTA) at pH 6.0. Anomalous results were obtained in one of the

early tracer experiments due to the presence of carrier and radioactive iron in different chemical forms. Analytical tests at Long Ashton on the sample of Fe^{59}-labelled ferric chloride obtained from the U.K.A.E.A., Harwell, showed that whereas all the Fe^{59} was present as ferric iron, most of the carrier was present in a form which did not react with α,α-dipyridyl. Dr. Cook of Harwell informed us that Fe^{59} of high specific activity was prepared by bombarding iron oxide, which was then dissolved in concentrated hydrochloric acid, evaporated to dryness and taken up in water (pH 2). The calculated amount of carrier as solid $FeCl_3$ was then added to the solution of active iron which was made to volume with water. He suggests that in this instance the carrier iron was probably colloidal and did not exchange with the active iron present as the chloride. This was avoided in all later samples by making the solutions containing active and carrier iron strongly acid, before evaporating and taking up the residue in water.

RESULTS

Washing tomato "tops"

Ten tomato plants were sampled and washed in 0.3% "teepol" solution for 10 sec and subsequently rinsed in distilled water for 10 sec. A further 10 plants were left unwashed as controls. The iron contents of the tops are given in Table I.

TABLE I

The iron contents of tomato tops; (mean of ten replicates)		
Treatment	Unwashed	Washed in 0.3% "teepol"
Fe (μg/g dry weight)	166	88

The reduction of the Fe content of the plants after washing is significant at the 0.1% level. There were no significant effects on the P_2O_5 or K_2O contents.

In the next experiment the following treatments were given to 10 plants per group (*a*) no washing, (*b*) washed in 0.3% "teepol" for 10 sec then rinsed twice in distilled water for a total time of 20 sec (*a*) as for (*b*) plus a further wash in 0.25 *N* HCl for 10 sec followed by two 10 sec rinsings in distilled water. The iron contents of the tomato 'tops' are given in Table II.

Thus the Fe contents of the plant 'tops' are significantly reduced by washing in either detergent or detergent and HCl. Further loss of Fe by the additional HCl treatment (*c*) is significant at the 5%

TABLE II

The iron contents of tomato 'tops' (mean of 10 replicates)			
Treatment	(*a*) Unwashed	(*b*) Washed in 0.3% "teepol" only	(*c*) As for (*b*) and in 0.25 *N* HCl
Fe (μg/g dry weight) . . .	165	115	88

Reduction in Fe content by "teepol" wash (*b*) or HCl wash (*c*) significant at 0.1% level.
Difference in Fe content between detergent wash (*b*) and detergent + HCl wash (*c*) significant at 5% level only.

level only. There was no change in the P, K and Mn contents of the plants as a result of the wash treatments.

In the next experiment 20 plants were grown in a dustproof chamber and the same number in the glasshouse; 10 plants from the glasshouse and the same number from the chamber were washed in detergent for 20 sec followed by two rinsings in distilled water for 20 sec. The results are given in Table III.

TABLE III

The iron contents of tomato 'tops' (μg/g dry weight; mean of 10 replicates)		
Location	Unwashed	Washed in "teepol" and water
Glasshouse	275	116
Dustproof chamber	100	85

Reduction of Fe content of washed plants grown in glasshouse significant at the 0.1% level.
Reduction of Fe content of washed plants grown in the dust proof chamber *N.S.*

Thus it appears that washing has reduced the iron content of plants grown in the glasshouse but not of those grown in a dustproof chamber.

Duration of washing treatment

The effect of the duration of washing on the iron content of tomato 'tops' was next investigated. The plants were washed in the detergent solution, drained for 5 sec and then rinsed in distilled water. The results are shown in Fig. 1; half of each time interval was used for the detergent wash and the remainder for the water rinse.

Significant amounts of iron were removed during the first 20-sec washing period but additional treatment did not further reduce

iron. The Mn content of the plants, which is similar to the iron status was unchanged and K, which is more readily leached than other nutrients, was also unaffected by this treatment.

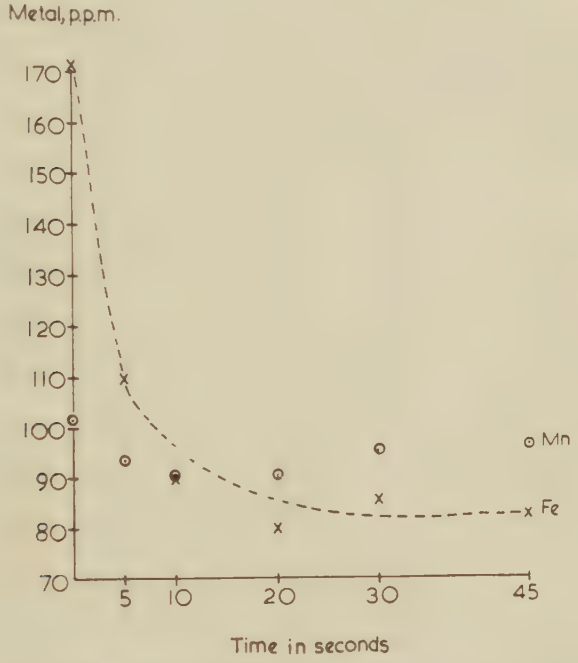

Fig. 1. Iron and manganese contents of tomato leaves after washing them for various periods in 0.3 per cent 'teepol' and distilled water.

Washing chlorotic plants

It was of interest to determine whether the iron content of iron-deficient leaves was also reduced after the washing treatment. An iron-type chlorosis was produced by adding three levels of either Co or Ni sulphates to the soil when the plants were 9 weeks from seed. Iron-chlorosis effects appeared within a week of the metal application and the plants were sampled at that stage. The normal and chlorotic leaves were washed in the detergent for 10 sec followed by two 10-sec rinsings in distilled water. The results are given in Table IV.

Iron chlorosis appeared in the young leaves of all plants given metal treatment. The data show that washing the leaves has removed a significant amount of iron from the leaf surfaces of both normal and chlorotic plants grown in the glasshouse. The iron

content of the washed leaves reflects the iron-deficiency symptoms produced by the heavy-metal treatments.

TABLE IV

The effect of the wash treatment on the iron content of normal and chlorotic leaves. (Mean values for Fe in μg/g dry weight)

Factor \ Location	Glasshouse		Dustproof chamber	
Cobalt * treatment				
Washed	84.8	signif. at	81.9	N.S.
Unwashed	93.1	0.1%	87.9	
Co_0 *	104.8		102.9	
Co_4	84.2		77.5	
Co_8	78.0		74.5	
	$Co_0 > Co_4$ & Co_8 at 0.1%		$Co_0 > Co_4$ & Co_8 at 0.1%	
†Significant Differences 5%	5.6		10.93	
1%	7.45		14.55	
0.1%	9.75		19.04	
Nickel * treatments				
Washed	131.5	signif. at	61.4	N.S.
Unwashed	109.0	0.1%	64.4	
Ni_0 *	143.0		77.0	
Ni_8	112.3		56.2	
Ni_{16}	105.6		55.7	
	$Ni_0 > Ni_8$ & Ni_{16} at 0.1%		$Ni_0 > Ni_8$ & Ni_{16} at 0.1%	
†Significant Differences 5%	8.3		5.1	
1%	11.1		6.8	
0.1%	14.6		8.9	

* Metal treatments are milliequivalents of cobalt or nickel sulphates per pot containing 1.8kg of soil.

† Average of washed treatments.

Experiments in solution culture

Tomato plants were grown from seed in a culture solution freed from iron to which 1 mg iron as Fe-EDTA labelled with 4 μC of Fe^{59} per litre was added. Six, nine and twelve weeks after seed germination the 'tops' were washed, dried and the iron contents of the wash solutions determined by chemical and tracer methods. The results of two separate experiments are given in Tables V and VI.

The results show that washing plant 'tops' for periods between 30 and 90 sec in "teepol" and water resulted in a large loss of iron from the external surfaces. The low radioassay values for Fe^{59} in

TABLE V

Effect of washing with "teepol" and water on the removal of iron from tomato plants

Leaf washing * procedure: sequence and duration	(1) μg Fe leached from plant 'tops' determined by radioassay	(2) (1) as per cent total Fe in plant 'tops'	(3) μg iron in the wash solutions, determined by chemical methods (surface iron)	(4) (3) as per cent total Fe in plant 'tops'
Experiment 1 (6 weeks after germination)				
A (10 sec)	0.24 ± 0.05	0.2	87	51
C (10 sec)	0.10 ± 0.08			
C (10 sec)	< 0.01			
Experiment 2 (9 weeks after germination)				
A (20 sec)	0.37 ± 0.06	0.3	86	33
B (20 sec)	0.27 ± 0.05			
C (20 sec)	0.09 ± 0.06			
Experiment 3 (12 weeks after germination)				
A (30 sec)	0.68 ± 0.08	0.6	74	31
C (30 sec)	0.25 ± 0.07			
C (30 sec)	0.17 ± 0.06			

* Wash solutions: — A = 0.3 per cent "teepol" in distilled water.
B = 0.15 per cent "teepol" in distilled water.
C = distilled water.

the wash solutions show there was no appreciable loss of iron from the internal plant tissues. The greatest amount of Fe^{59}, although small compared with the total amounts in the leaf, appeared in each initial wash. This may have resulted from the abrasion of glandular hairs during washing.

The results in Table VI also show that iron determined by chemical or by tracer techniques are in close agreement, and are within the limits of error for the methods, viz ± 2% radioassay, ± 5% chemical method.

In these experiments the recovery of iron in the plants (roots and tops) and in the culture solution, determined by radioassay and chemical methods, was in close agreement with the calculated amount of iron added, viz 1 mg Fe per culture flask.

TABLE VI

The effect of washing with "teepol" and water on the removal of iron from tomato 'tops'

Time after germination (weeks)	Total * washing time (sec)	(1)	(2)	(3)	Total Fe (μg) in 'tops'	
		μg Fe leached from tops determined by radioassay	(1) as per cent total Fe in 'tops'	μgFe in wash solutions determined chemically	Radio-assay method	Chemical method
6	30	A 0.76±0.08 B 0.58±0.09 C 0.61±0.07	0.4	103	462	455
6	90	A 1.01±0.09 B 0.38±0.07 C 0.27±0.02	0.5		341	345
9	30	A 0.46±0.07 B 0.29±0.05 C 0.25±0.07	0.3	69	275	286
9	60	A 0.78±0.08 B 0.20±0.07 C 0.29±0.06	0.3	49	442	470
9	90	A 0.91±0.08 B 0.22±0.09 C 0.24±0.05	0.3	64	436	465

* Equally divided between washes A: 0.3 per cent "teepol" in distilled water.
B: distilled water.
C: distilled water.

Recovery of tracer iron from plant digests

Iron labelled with Fe^{59} was added to dried and ground leaves of apple, wheat, and cauliflower respectively: — (*a*) before acid digestion, (*b*) at the hydrolysis stage, when the silica had been dehydrated. After the digests were completed, they were analysed for total iron by the chemical method and for Fe^{59} by radioassay. In a similar experiment the recovery of iron from acid digests of washed and unwashed tomato leaves was determined. The results are given in Tables VII and VIII.

The chemical and radioassay results indicate that iron is fully recovered from the acid digests irrespective of the plant material used or the stage at which the iron was added.

DISCUSSION

The presence of iron as a contaminant on foliage, and washing techniques for its removal in detergent and water or dilute acid,

TABLE VII

The recovery of Fe^{59} from acid digests of dried plant material

Treatment (Addition of 200 μg Fe labelled with 0.05 μC Fe^{59})	Apple		Wheat		Cauliflower	
	Fe μg/g dry weight Chemical Method	Fe^{59} per cent recovery	Fe μg/g dry weight Chemical Method	Fe^{59} per cent recovery	Fe μg/g dry weight Chemical Method	Fe^{59} per cent recovery
Fe only — no plant material	200	98.4				
	203	106.0				
	203	100.5				
Plant material only — no Fe added	165		33		391	
	163		36		399	
	159		33		386	
Fe added to plant material prior to acid digestion	161	98.5	33	106.0	396	100.5
	160	101.2	33	95.5	403	100.4
	157	101.0	34	99.0	392	102.3
Fe added to plant material after dehydration of silica, at hydrolysis stage	162	105.0	35	103.0	396	100.9
	159	97.4	34	103.2	399	106.2
	162	99.1	34	98.8	420	103.1

The statistical precision of counting was such that any values for Fe^{59} per cent recovery between 94–106 may be accepted, there is thus no evidence of any loss of Fe^{59} from the digests.

TABLE VIII

The recovery of Fe^{59} from acid digests of tomato leaves

Treatment (Addition of 109 μg Fe labelled with 0.12 μC Fe^{59}	Fe μg/g dry weight		Percent recovery of Fe^{59}	
	Washed in "teepol" and water	Unwashed	Washed in "teepol" and water	Unwashed
Fe only – no plant material			99.0	
			99.6	
			98.7	
			98.0	
Plant material only – no Fe added	61	64		
	63	64		
	61	64		
	62	68		
Fe added to plant material prior to acid digestion	62	64	98.1	102.5
	60	64	99.3	104.9
	62	63	100.4	103.1
	62	66	99.1	101.0
Fe added to plant material after dehydration of silica, at hydrolysis stage	62	64	100.6	99.6
	61	64	102.2	100.5
	61	66	102.7	100.8
	61	64	101.6	98.3

have been reported (Jacobson[4], Smith, Reuther and Specht[15], and Mason[6]). In these investigations, however, it was not definitely established whether iron was leached or not from within the tissues during the washing.

We have shown that in tomato grown in a glasshouse, iron is a serious contaminant on plant surfaces and that it can be removed completely by washing the foliage in 0.3% "teepol" detergent in water, without loss of nutrient from the internal tissues. Tracer iron was used to show that the element was removed completely from the external surfaces without loss from inside the plants.

It has been suggested that the adsorption of iron on silica and the formation of soluble iron complexes during acid digestion of plants may also contribute to the erratic iron results (Mason[5]). Our experiments, however, show that the tracer and inactive iron were fully recovered when added at various stages during the acid digestion of dried plant materials, which had a wide range of silica contents. Further, the iron content of tomato 'tops' grown in solution culture containing iron labelled with Fe^{59} and given the wash treatment, was similar whether determined by radioassay or chemical analysis. Thus even should iron complexes form during acid digestion they did not interfere with the chemical analysis of iron in tomato.

The iron contents of subsamples of washed leaves of tomato were less variable than those for unwashed leaves.

SUMMARY

1. Iron which is shown to be present as a contaminant on leaf surfaces of tomato can be removed completely by washing the leaves for 30 sec in 0.3% "teepol" and water followed by a 30 sec rinsing in distilled water.

2. By using radioactive iron it is shown that there was no loss of the metal from within the plants during leaf washing. Agreement between chemical and radioassay data for iron showed that the metal was completely removed by the wash procedure.

3. In tomato grown in the glasshouse consistent differences between the iron contents of normal and chlorotic leaves were obtained after leaf washing. When the plants were grown in a dustproof chamber differences in iron status of green and chlorotic leaves were obtained without washing.

4. Added radioactive and carrier iron was fully recovered from acid digests of a variety of plant material, specially chosen to represent a range of silica contents.

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A COMPARISON OF THE EFFECT OF COMBINED NITROGEN ON NODULATION IN NON-LEGUMES AND LEGUMES

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As pointed out previously [4] evidence is accumulating that the non-legume root nodule plants play a significant part in connection with the biogeochemistry of nitrogen *. Their habitat requirements and thus their distribution tend to be completely different from those of legumes. For example *Myrica gale* inhabits peat bogs, *Alnus glutinosa* likewise favours swampy situations, *Shepherdia canadensis* is a scrub-plant extending to sub-arctic regions, while *Casuarina equisetifolia* occurs as a strand plant in the Indian and Pacific Ocean regions. In view of the nitrogen-fixing properties which have been demonstrated for the nodules of most of the genera, it cannot be doubted that these plants play a part in the maintenance of soil nitrogen. Thus the study of factors liable to affect the development or the activity of the nodules is of interest.

In the present instance a study has been made of the effect of the presence of combined nitrogen in the rooting medium on the development of nodules in *Myrica* and *Alnus*. It is well known that like free-living fixers such as *Azotobacter*, the nodulated legume uses combined nitrogen in preference to free nitrogen, and that this is accompanied by a reduced development of nodules. Large amounts of combined nitrogen, in excess of those likely to be present naturally in soils, are required to produce complete suppression of nodules. Thus it is a matter of common observation that broad beans and peas show abundant nodulation in the garden even though the soil has been well manured or fertilised.

* Apart from the legumes the following Angiospermous genera also form root nodules inhabited by a symbiotic micro-organism: *Myrica*, *Alnus*, *Hippophaë*, *Elaeagnus*, *Shepherdia*, *Casuarina*, *Coriaria*, and *Ceanothus*.

Some previous observations on the effect of this factor in non-legumes are available. Bjorkman [1] grew alders in a humus-sand mixture and observed that in the presence of 100 mg ammonium-nitrate nitrogen per litre of culture solution the volume of nodules formed was reduced from 33 cu.mm per plant (the value with no added nitrogen) to 15 cu.mm. Nodulation was almost completely suppressed in the presence of 200 or 400 mg nitrogen per litre. Bond, Fletcher and Ferguson [3] reported on the effect of up to 100 mg ammonium nitrogen per litre on nodulation chiefly in *Alnus* and *Hippophaë*. In *Alnus* after 12 weeks' growth the absolute weight of nodules per plant was greater at all nitrogen levels than with zero nitrogen, though relative to the increased weight of the plants the nodule development was smaller.

Nodulation in *Hippophaë* was found to be much more sensitive. Quispel [5] reported rather different findings for *Alnus*. After 7 weeks' growth a level of approximately 5 mg ammonium nitrogen per litre was found to have reduced the absolute weight of nodules per plant to half, while 50 mg practically suppressed nodulation. These are much greater effects than those observed by the previous authors.

In the present work the case of *Alnus* has been further investigated, particularly in that the effect of feeding the plants with combined nitrogen prior to inoculation has been tested, thus approaching more nearly conditions in the field. *Myrica gale* also has been studied, and for purposes of comparison a similar experiment with a legume (*Ulex europaeus*) has been included.

METHODS

Seedlings were raised in peat (*Myrica gale*), sand (*Alnus glutinosa*) or on mineral salt agar (*Ulex europaeus*), the seed of the last species being surface-sterilised prior to sowing. The seedlings were transferred into water culture in Crone's nitrogen-free solution adjusted to a favourable pH, the containers being 2-litre glazed earthenware jars each with 5 or 6 plants supported in teak tops. Combined nitrogen was added as desired in the form of ammonium sulphate. To inoculate the non-legume the appropriate nodules were ground up in water (3.5 g for *Myrica* and 20 g for *Alnus* per 100 ml water), the suspension so obtained being applied to the roots of the young plants. With *Ulex* a similar suspension was prepared from a pure culture of an effective strain of rhizobium. In certain instances the plants were allowed to grow for a period in the presence of combined nitrogen prior to inoculation.

In experiments of this type involving the utilisation of ammonium nitrogen it is of critical importance that the pH of the culture solution, which of course tends to fall, should be frequently restored to its proper level. The pH was tested every second day, and when necessary was restored to the correct value by the addition of an appropriate amount of sodium hydroxyde, determined by titration of a small sample of the culture solution. The ammonium-nitrogen content of the culture solution was also determined periodically and further amounts added as necessary to maintain the desired level. The culture solution was completely renewed every 3 to 4 weeks.

DATA

Myrica gale

Young plants with 3 or 4 leaves were set up in water culture on 22nd May at pH 4.5. Four levels of combined nitrogen were established in different jars on the same day, viz, 0, 10, 50, and 100 mg nitrogen per litre, and inoculation was effected 2 days later The first nodules appeared 2 weeks later. Considerable losses of plants occurred, which in subsequent unpublished work Miss I. C. Gardner has shown can be entirely obviated by using a more dilute form of Crone's solution. Thus the numbers of plants available for harvest on 28th August were rather small. Typical plants are shown in Plate Ia and the harvest data are provided in Table I. Plant growth was much stronger in the presence of combined nitrogen and it will be observed that nodule weight was also greatly increased, though relative to the dry weight of the plant as a whole (see right-hand column) nodule weight was depressed. An analysis of variance shows that the fall from 12.2 to 5.4 is highly significant, but that the subsequent further falls are not significant at $P = 0.05$.

TABLE I

Effect of added combined nitrogen on nodulation and growth of *Myrica* plants *

Mg NH_4-N added per litre of culture soln.	Mean height of shoot in cm	Mean dry weight per plant in mg		Mean value for nodule weight as percentage of whole plant weight
		Nodules	Whole plant	
0	3	4	33	12.2
10	8	17	304	5.4
50	12	24	561	4.2
100	10	19	488	3.6

* The numbers of plants harvested at the different nitrogen levels, starting with zero nitrogen, were: 9, 12, 6, and 5 respectively.

Alnus glutinosa

The experiment comprised two parts. With one series of plants the differential dosage with combined nitrogen was commenced directly after transplanting into water culture, the plants being at the 1 to 2 leaf stage with an over-all height of 3 to 4 cm. Inoculation was effected the following day. With the other series the plants were grown on in water culture in the presence of 50 mg ammonium nitrogen per litre for a period of 5 weeks prior to the commencement of the differential treatment with nitrogen and to inoculation. By this time the plants had 4 to 5 leaves and an over-all height of 7 to 9 cm. The procedure was so timed that the plants of both series were all inoculated on the same day (30th July) with the same inoculum. Analysis of similar plants showed that the percentage nitrogen content (dry matter basis) at the time of inoculation was 1.3 for the plants of the first part of the experiment and 3.4 for those of the second. The first nodules appeared only 10 days after inoculation. Growth was allowed to continue for approximately 10 weeks, the pH of the culture solution being maintained near to 6.3.

Typical plants of the first series are shown in Plate Ib, while the harvest data are given in the upper part of Table II. It will be observed that as in *Myrica* the growth of the plants was again much benefited by the presence of combined nitrogen. The dry weight of nodules is higher in the presence of 10 mg nitrogen per litre than in zero nitrogen, but is smaller when 100 mg is present. Relative to the whole plant weight, nodule weight falls sharply between the 0 and 10 mg nitrogen levels (see right-hand column), and thereafter tends to fall more slowly.

Data for the second series of plants, given pre-treatment with nitrogen prior to inoculation, are shown in the lower part of Table II. These plants were naturally much larger than those of the first series. It will be noted that the dry weight of nodules is approximately doubled by the presence of 10 mg nitrogen per litre, but at higher nitrogen levels is much reduced, so that with 100 mg nitrogen present the dry weight is only one quarter of that in nitrogen-free solution. Thus in this respect the effect of combined nitrogen is considerably more marked than in the first series. The right-hand column shows that pre-treatment with nitrogen reduced the subsequent nodulation in nitrogen-free solution, since the value of 3.4 per cent is to be compared with 5.3 per cent in the upper part of

the Table. The presence of combined nitrogen after inoculation depressed this value further.

TABLE II

Experiment with *Alnus* *				
Procedure	Mg NH_4-N added per litre of culture solution **	Mean dry weight per plant in mg		Mean value for nodule weight as percentage of whole plant weight †
		Nodules †	Whole plant	
First Series				
Differential treatment with nitrogen and inoculation applied directly after transplanting into water culture	0	3.3	65	5.3
	10	5.5	394	1.7
	50	3.6	436	1.0
	100	2.6	403	0.7
Second Series				
Plants all supplied with uniform combined nitrogen for 5 weeks prior to start of differential treatment and inoculation	0	28	878	3.4
	10	54	2275	2.4
	50	20	2363	0.8
	100	7	2116	0.4

* 15 plants were harvested at each nitrogen level in the first series and 12 in the second series.

** The maximum deviations from the desired levels of NH_4-N during the growth period were as follows: — 10 mg fell to 6 mg, 50 to 47 mg, 100 to 95 mg.

† Differences required for significance ($P = 0.05$) are as follows; — Nodule Dry Weight, 0.9 mg (first series) and 10 mg (second series); Nodule Weight as percentage of Whole Plant Weight, 0.8 (first series) and 0.3 (second series).

In the above experiments no counts were made of the numbers of nodule clusters formed in the different treatments, but it was obvious from inspection of the plants that the beneficial effect of the lower levels of combined nitrogen on nodule weight was due more to increase in size of the nodule clusters than to increase in their total number.

A

B

Plate I.

A. Nodulated plants of *Myrica gale* after 14 weeks' growth in water culture, supplied with (left to right) 0, 10, 50 and 100 mg NH_4-N per litre. The upward-growing roots spring from the nodule lobes.* (× 1/3).

* Note that while the photograph suggests that plants supplied with 100 mg N per litre were superior to those supplied with 50 mg, the harvest data subsequently obtained (Table I) revealed no significant difference in the mean growth of these plants.

B. Nodulated plants of *Alnus glutinosa*, first series, after 10 weeks' growth in water culture, supplied with (left to right) 0, 10, 50 and 100 mg NH_4-N per litre. (× 1/5).

A

C

Plate II.

A. Young plants of *Ulex europaeus* the time of inoculation, plant fr first series on right, from second left. (× 1).

B. Nodulated plants of *Ulex* (first ser after 10 weeks' growth in wa culture, supplied with (left to rig 0, 10, 50, 100 and 150 mg NH per litre. (× 1/7).

C. Nodulated plants of *Ulex* (sec series) after 10 weeks' growth in wa culture, supplied with (left to rig 0, 10, 50 and 100 mg NH_4-N litre. (× 1/6).

Ulex europaeus

The general plan of the experiment was as in *Alnus*. The two series of plants, the first newly transplanted into water culture, the second grown for 3 weeks in the presence of 50 mg combined nitrogen per litre, were provided with different levels of combined nitrogen on 22nd May and inoculated the following day. Typical plants at this stage are shown in Plate IIA. Analysis of similar plants showed percentage nitrogen contents on the dry matter basis of 7.4 for plants of the first series and 4.1 for those of the second. All plants were nodulated within a fortnight. Growth was allowed to continue for about 9 weeks at pH 6.3.

TABLE III

Experiment with *Ulex* *					
Procedure	Mg NH_2-N added per litre of culture solution **	Mean number of nodules per plant †	Mean dry weight per plant in mg		Mean value for nodule weight as percentage of whole plant weight †
			Nodules †	Whole plant	
First Series					
Differential treatment with nitrogen and inoculation applied directly after transplanting into water culture	0	38	11	166	6.6
	10	82	10	304	3.4
	50	69	9	342	2.8
	100	95	8	409	2.1
	150	79	8	355	2.3
Second Series					
Plants all supplied with uniform combined nitrogen for 3 weeks prior to start of differential treatment and inoculation	0	97	12	200	6.1
	10	148	16	468	3.6
	50	76	8	600	1.3
	100	60	6	544	1.1

* The number of plants harvested varied from 17 to 21 at each treatment.

** The maximum deviations from the desired levels of NH_4-N during the growth period were as follows; — 10 mg fell to 5 mg, 50 to 40 mg, 100 to 87 mg, 150 to 140 mg.

† Differences required for significance ($P = 0.05$) are as follows: — Nodule Number, 34 (first series) and 22 (second series); Nodule Dry Weight, 2 mg in both series; Nodule Weight as percentage of Whole Plant Weight, 0.7 (first series) and 0.6 (second series).

Typical plants at harvest of the first series are shown in Plate IIb, and the corresponding data in the upper part of Table III. It will be noted that plant dry weight was about doubled in the presence of combined nitrogen, but this was not accompanied by an increase in nodule weight. The latter declined in the presence of 50 mg or more of nitrogen. Nodule number was doubled by the change from zero nitrogen to 10 mg per litre, and remained at this high level with further increase in nitrogen. Obviously the nodules were smaller in weight and size in the presence of nitrogen. Calculation from the data presented shows that the mean dry weight per nodule diminished from 0.29 mg in zero nitrogen to 0.10 mg in the 150 mg level of nitrogen. Nodule weight as a percentage of whole plant weight (right-hand column) shows a marked fall in the presence of 10 mg nitrogen, and thereafter falls more slowly.

Plants of the second series are illustrated in Plate IIc, and their data presented in the lower part of Table III. In most respects combined nitrogen now had a more adverse effect on nodulation than in the first series. Thus nodule weight and number, and nodule weight as a percentage of whole plant weight, were all more drastically reduced in the presence of 50 or 100 mg nitrogen per litre than in the first series. However with 10 mg nitrogen present nodule weight per plant was significantly increased, and mean dry weight per nodule fell only from 0.12 mg in zero nitrogen to 0.10 mg in 100 mg nitrogen per litre. These findings will be further discussed below.

DISCUSSION

The results of the *Alnus* experiment (first series) may be compared with those of the similar experiment of Bond, Fletcher and Ferguson [3]. Both agree in showing that as the amount of combined nitrogen supplied is increased, nodule weight falls continuously relative to the weight of the plant as a whole, but that at low levels of nitrogen the absolute weight of nodules is substantially increased. In the experiment of Bond *et al.*, with 100 mg nitrogen supplied nodule weight was still greater than in zero nitrogen, while in the present experiment this was only true up to some nitrogen level between 10 and 50 mg per litre. Other experience shows that the reason for this difference is that the present experiment was started a month later in the growing season, when conditions are less favourable to nodule development.

The increases in absolute weight of nodules noted in the above and other experiments are obviously related to the increased growth of the plant as a whole in the presence of combined nitrogen. The nodules share in this greater growth, but not to a proportionate extent, *i.e.* the combined nitrogen is already exercising a differential effect on nodule growth on one hand and on root and shoot growth on the other. The enhanced growth of the plant in the presence of combined nitrogen arises because of the time required for the nodules to develop and become functional, the growth of plants in nitrogen-free solution being greatly handicapped during this period. In longer-term experiments the growth of the latter plants would probably gradually overtake that of those supplied with combined nitrogen and as this happened the superiority in absolute nodule weight would diminish. Thus the effect is probably a temporary one.

As noted already, when the commencement of differential treatment with nitrogen and inoculation were delayed until the *Alnus* plants had grown to a considerable size with the help of combined nitrogen, as in the second series, then the unfavourable effect of the higher levels of nitrogen on nodule development were more pronounced. The procedure in this part of the experiment was generally similar to that employed by Quispel [5], but there are marked differences in result since, as noted earlier, in his experience ammonium nitrogen at only 5 mg per litre had an entirely depressing effect on nodule formation. Although it is clear that in most respects Quispel's experiments were carefully carried out, it is not quite certain that his technique excluded the possibility of the pH falling to levels harmful to nodulation. Thus he used a solution of inferior buffering power to Crone's, a high ratio of number of plants per unit volume of solution, and corrected the pH only once weekly.

Myrica gale obviously resembles *Alnus glutinosa* closely as regards the effect of combined nitrogen on nodule development. Thus as the nitrogen level is increased the weight of nodules per plant at first rises and subsequently falls, while relative to whole plant weight the value for nodules is continuously depressed.

Besides their immediate value in providing a basis of comparison for the non-legumes, the *Ulex* data are of wider interest in that, unlike many previous data for legumes, they were obtained by a technique permitting close control of the level of combined nitrogen and of pH. At the levels used, combined nitrogen did not exert a partic-

ularly drastic effect on nodulation. Thus in the first series, levels of nitrogen clearly in excess of the requirements of the plants (under favourable growth conditions) resulted in increased nodule numbers, a rather slight reduction in nodule weight per plant, and reductions to about one third in the weight of nodules relative to that of the whole plant and in the mean weight per nodule. Adverse effects were somewhat stronger after pre-treatment with combined nitrogen. It is important to restate that in this experiment the first series of plants showed a higher percentage of nitrogen (7.4) at the time of inoculation than those of the second series (4.1). It has been shown by previous workers that in legumes the carbohydrate-nitrogen ratio within the tissues is an important factor in the control of nodulation. A high ratio usually tends to favour nodulation. This is undoubtedly the reason why in zero nitrogen the percentage nodule weight was not considerably lower in the second series than in the first, as was the case in *Alnus*.

For the comparison of *Alnus* and *Ulex* it will be best to confine attention chiefly to the second series of plants of each experiment in view of the large disparity in the nitrogen balance of the plants at the time of inoculation in the first series, for as noted already the percentage nitrogen in *Alnus* was 1.3, but in *Ulex* was 7.4. This is due chiefly to differences in seed composition. By the time of inoculation in the second series of plants the difference had largely disappeared, the percentage nitrogen having increased to 3.4 in *Alnus* and decreased in *Ulex* to 4.1. Reference to the second series of Tables II and III reveals many similarities in response to combined nitrogen. With 10 mg nitrogen present nodule weight per plant is increased in both species, though the increase is smaller in *Ulex* than in *Alnus*. At higher levels of nitrogen nodule weight falls. In both species nodule weight relative to plant weight falls continuously. Thus *Alnus* resembles *Ulex* closely in its responses to combined nitrogen, and it is clear that the same is true of *Myrica*, although the data for it are less extensive.

This article is intended to be essentially factual in type and the possible explanation of the observed effects of combined nitrogen on nodule development will not be considered in detail. A full discussion of suggested explanations for legumes is provided by Wilson [6]. It is reasonable to suppose that the mechanism of the combined nitrogen effect in non-legumes is similar to that in legumes.

The data reported for *Alnus* indicate that the combined nitrogen exerts its effect internally in the plant tissues. Thus the effect was greater when the tissues were already relatively rich in nitrogen as a result of pre-treatment (second series).

Indirectly the present data confirm that *Hippophaë* is much more sensitive to combined nitrogen than *Alnus* or *Myrica*. Bond, Fletcher and Ferguson [3] reported that in the first genus the presence of only 5 mg nitrogen per litre greatly decreased nodulation, but they pointed out that this referred to plants which had been grown for a period in the presence of combined nitrogen to inoculation. The present experiments show that *Alnus* similarly treated is still much less sensitive to combined nitrogen than is *Hippophaë*.

This article is not concerned with the question of how active in fixation are the nodulus formed in the presence of combined nitrogen. On the basis of isotopic tests with *Alnus* and *Myrica*, Bond [2] concluded that such nodules fix appreciable quantities of free nitrogen.

The data presented indicate that the nodulation of *Alnus* and *Myrica* in the field will not be seriously reduced by the levels of combined nitrogen likely to be present in the normal habitats of the plants.

SUMMARY

1. The effect of the presence of combined (ammonium) nitrogen in the rooting medium on nodule development has been investigated in *Alnus glutinosa* and *Myrica gale*, the plants being grown by a water culture technique with provision for the control of the level of combined nitrogen and of pH. For purposes of comparison a similar experiment with a legume (*Ulex europaeus*) has been included.

2. In some instances the differential treatment with combined nitrogen was commenced and inoculation effected at an early stage in plant development, while in others the plants were grown on to a larger size before these treatments were applied.

3. In the two non-legumes the presence of combined nitrogen led to an increase in the weight of nodulus formed per plant, at least at the lower levels of nitrogen. Relative to the enhanced growth of the plant as a whole, nodule development was continuously depressed as the level of combined nitrogen was increased.

4. In *Ulex* when the nitrogen status of the plants at the time of inoculation was similar to that in the corresponding non-legume experiment, the above responses were also shown by the legume. It is concluded that *Alnus* and *Myrica* resemble legumes closely in their responses to combined nitrogen.

5. The data show that in *Alnus* the effect of a given level of combined nitrogen on nodulation depends on the nitrogen status of the plant at the time of inoculation, the adverse effect tending to be stronger if the plant is initially relatively high in nitrogen. This suggests that as in legumes the effect of the combined nitrogen is exerted internally in the plant tissues.

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THE SOLUTION OF MANGANESE OXIDE BY IRON SOURCES USED IN NUTRIENT SOLUTIONS

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INTRODUCTION

Recently Boken [2,3] reported that the availability to plants of native and applied manganese was increased when ferrous sulphate was added to soil and she suggested that the ferrous ion either reduced soil manganese oxides or prevented the added manganese from being transformed into less available compounds.

The present author has been examining the availability of biologically precipitated manganese oxide in sand cultures using nutrient solutions. It became obvious that the choice of the iron source in the nutrient solution was important, firstly because many of the substances used to stabilize iron can complex with manganese, and secondly, because Boken's work suggested that ferrous iron may reduce manganese oxide. Both these reactions could influence the availability of the manganese oxide.

This report describes experiments which were undertaken to determine suitable iron sources for the study of the availability of manganese oxide in sand cultures. The extent of reduction of manganese oxide by ferrous ion was also examined in order to test Boken's explanation.

METHODS

Manganese oxide, containing 0.25 or 1.5 mg Mn, was suspended in 50 ml of various solutions and allowed to stand for 18 h at 20°C after which the magnanese in 25 ml of the filtered supernatant was determined colorimetrically as permanganate. The solutions tested were ferrous sulphate, ferric chloride, tartaric acid, citric acid, disodium ethylenediaminetetra acetate

(Na_2-EDTA), sulphuric acid and equimolar mixtures of the iron solutions and the acids. The solutions were tested at concentrations of 5×10^{-5}, 10×10^{-5} ans 15×10^{-5} molar. pH was determined with a glass electrode.

EXPERIMENTAL RESULTS

Arnon [1] recommended ferrous tartrate as the iron source for plant culture solutions. The solubility of biologically precipitated manganese oxide (Bromfield [4]) was therefore measured in mixtures of ferrous and tartrate solutions, and for comparison, in ferrous sulphate and tartaric acid solutions separately. The results of this experiment (Fig. 1) show that the oxide was soluble in ferrous + tartrate mixtures at a concentration as low as that used in Arnon's nutrient solution (point A on Fig. 1), and that it was also soluble

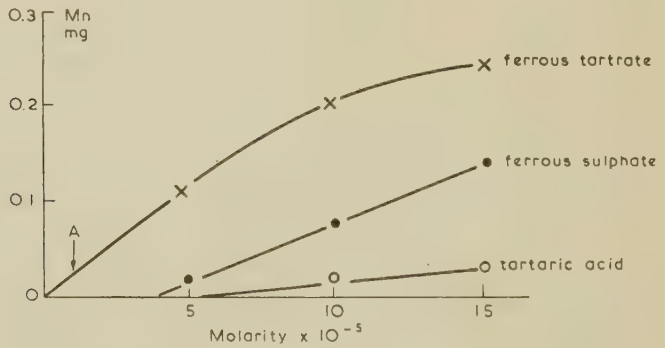

Fig. 1. Solution of biologically precipitated manganese oxide by 25-ml sample of tartaric acid, ferrous sulphate and ferrous tartrare at various concentrations after 18 h at 20°C.

in 5×10^{-5} *M* ferrous sulphate and in 10×10^{-5} *M* tartaric acid. It can be seen from Fig. 1 that the ferrous + tartrate mixture dissolved more manganese than did ferrous sulphate or tartaric acid alone. The flattening of the ferrous + tartrate curve is due to the oxide being completely dissolved at the higher concentrations. These results suggest that ferrous + tartrate would be an unsuitable iron source in manganese oxide availability studies; they also show that ferrous sulphate alone can dissolve appreciable amounts of biologically precipitated manganese oxide.

To test for other suitable iron sources the solubility of manganese oxide was measured in solutions of ferric chloride, ferric + tartrate, ferric + citrate, ferric EDTA; and for comparison in the free acids

and corresponding ferrous solutions (except Fe-EDTA). For these tests γ-MnO_2 * was used instead of biologically precipitated oxide because sufficient was available to use it in excess amounts and as Jones and Leeper [5] had found it available to plants. The solubility of γ-MnO_2 in the various solutions are given in Figs. 2 and 3.

Fe-EDTA dissolved manganese oxide only at relatively high

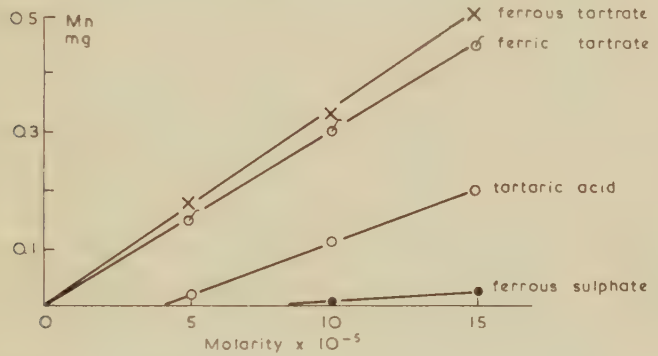

Fig. 2. Solution of γ-MnO_2 by tartaric acid, ferrous sulphate and ferrous and ferric tartrate as for Fig. 1.

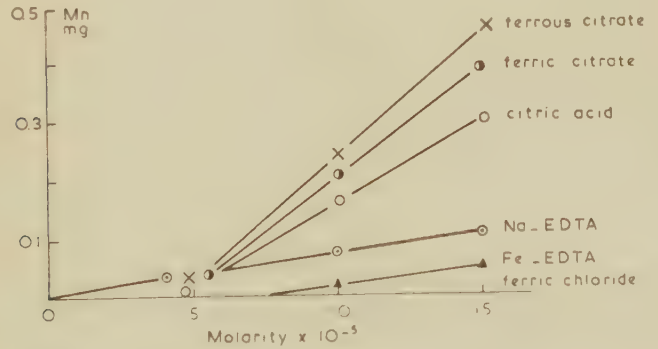

Fig. 3. Solution of γ-MnO_2 by citric acid, ferric chloride, ferrous and ferric citrate, ferric EDTA and Na_2-EDTA as for Fig. 1.

concentrations. (10×10^{-5} M) whereas very dilute Na_2-EDTA solutions ($< 5 \times 10^{-5}$ M) dissolved appreciable amounts of manganese. This suggests that Fe-EDTA might be a safe iron source provided there is no excess of free Na_2-EDTA.

* The sample of γ-MnO_2 was kindly donated by Dr. L. H. P. Jones.

Both ferric + citrate and ferrous + citrate, although they dissolved much manganese at concentrations above 5×10^{-5} *M*, may be suitable iron sources since there was no detectable solution at the concentrations required for adequate iron nutrition (1×10^{-5} *M*). The results with ferric + tartrate showed that this iron source is as unsatisfactory as ferrous + tartrate since both solutions dissolve manganese at low concentrations; ferric chloride on the other hand did not dissolve any measurable quantity of manganese even at 15×10^{-5} *M*.

Since manganese oxide becomes more oxidizing with increasing acidity, the effect of pH on the solubility of manganese oxide by ferrous solutions was examined. The pH values of 15×10^{-5} *M* solutions of ferrous sulphate and ferrous + tartrate were first determined, to see if there was a difference in pH which might account for the differences observed in Figs. 1 and 2. The values were found to be pH 5.0 and 3.8 respectively. The solubility of γ-MnO_2 was therefore measured in 15×10^{-5} *M* ferrous sulphate at pH 5.0 and 3.8. It was found that the amount of manganese dissolved increased from 0.01 mg at pH 5.0 to 0.06 mg at pH 3.8 and that this increase was due to the greater activity of the ferrous iron since a dilute sulphuric acid control at pH 3.8 did not dissolve any manganese. This increase however was not large enough to account for the difference between ferrous sulphate and ferrous + tartrate solutions (Fig. 2), so that tartrate must have played an important part in the solution.

The pH value of 3.8 for 15×10^{-5} *M* ferrous + tartrate is less than that for most plant nutrient solutions and soils and so a knowledge of the dissolving power of ferrous solutions at higher pH values would help in assessing the suitability of iron sources in manganese availability studies. The solubility of γ-MnO_2 was therefore studied at various pH values. These were obtained by adjusting the solutions with 0.01 *N* NaOH using a glass electrode. The amounts of manganese dissolved at various pH values by 15×10^{-5} *M* ferrous + tartrate (50 ml) are given below:

pH	3.8	5.2	5.9	7.2
Mn (mg)	0.5	0.11	0.03	0.02

The solvent action of ferrous + tartrate decreased rapidly as pH rose from 3.8 to 5.2 but even so it was still appreciable at pH 5.2. Above pH 6 the amount dissolved was small.

DISCUSSION

These studies clearly show that manganese oxide can be dissolved by very dilute iron solutions and that the solution is greatest at low pH values in the presence of citrate and tartrate and ferrous iron. In studies of the availability to plants of manganese oxide in sand cultures, care must therefore be taken in choosing the iron source for nutrient solutions, if interference due to iron is to be avoided. Whilst the results indicate that Fe-EDTA and ferric + citrate at concentrations less than 5×10^{-5} *M* might be suitable, it is suggested, that since these solutions can dissolve manganese oxide at higher concentrations, manganese-free ferric chloride would be a more suitable reagent to use.

It is of interest that ferric + tartrate is almost as effective as ferrous + tartrate at dissolving manganese oxide. Qualitative tests for ferrous iron however showed that tartaric acid reduces ferric iron which suggests that the ferrous iron so formed may account for this similarity.

The reduction of manganese oxides (especially biologically precipitated oxide) by ferrous sulphate (Fig. 1) at first supports Boken's [3] explanation that ferrous sulphate increases manganese availability because of the reduction of manganese oxide. The results of the present work, however, showed that ferrous + tartrate, which is more soluble at high pH values than ferrous sulphate, did not dissolve much manganese oxide above pH 6. Boken [2] showed also that the amount of manganese extracted from soil by *M* magnesium nitrate, with and without ferrous sulphate, decreased rapidly as the pH of the extracting solution increased, and that at pH 8 virtually no manganese was extracted by either solution. It is unlikely therefore that the addition of ferrous sulphate would reduce appreciable amounts of manganese oxide in the soils studied by Boken [3], some of which had an initial pH as high as 8, unless such additions (0.5 and 3 g $FeSO_4.7\ H_2O$) also lowered the soil pH and hence increase the reducing power of the ferrous sulphate and soil organic matter.

That very dilute solutions of common organic acids can dissolve manganese oxide, suggests that a similar simple solution by these or other dilute solutions may occur in the rhizosphere of plants and that this maybe one mechanism for the uptake of manganese from insoluble oxides.

SUMMARY

The solubility of two manganese oxides was measured in 5×10^{-5} to 15×10^{-5} *M* iron and organic acid solutions. The oxides were soluble in all the 15×10^{-5} *M* solutions tested except ferric chloride. The amount of manganese dissolved by mixtures of the iron and acid solutions was greater than the sum of that dissolved by the separate solutions. It was suggested that ferric chloride should be used as the iron source in critical studies of the availability of manganese oxides in sand cultures.

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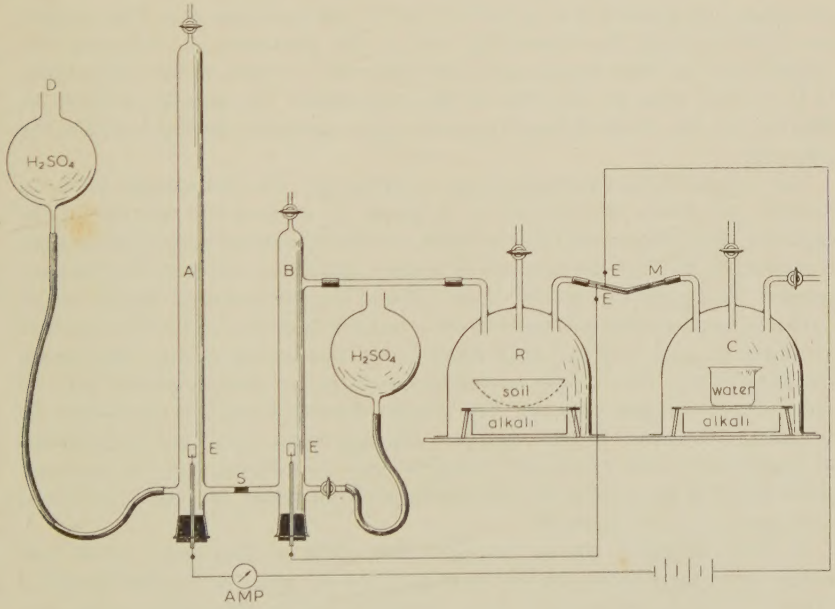
LETTER TO THE EDITORS

An Apparatus for the Determination of the Respiration Process in Soil Samples

A new apparatus was developed for the determination of respiration in soil samples. The carbon dioxide formed is absorbed in potassium hydroxide or in baryta and determined in the usual way, whereas the uptake of oxygen is estimated by a careful measurement of the decrease of the total volume of gas as a consequence of absorption of carbon dioxide by the alkali and of oxygen by the oxidative processes in the soil sample. The oxygen absorbed is replaced by electrolytically generated oxygen, while the hydrogen simultaneously formed is used as indication of the oxygen uptake.



In the container *R* (see figure) a petri dish, provided with a known amount of alkali, is covered by a basket of gauze, containing the soil sample. The container *R* is connected with another container *C* by a horizontal capillary tube, slightly bent downwards in its central part. The gas volume in vessel *C* is adjusted to exactly the same value as that of *R* by placing a beaker of water in *C*. The connecting tube is partly filled with mercury, and is provided with two electrodes. At the beginning of the experiment, the mercury level is adjusted between the two electrodes to be about 1 mm from the left one. The

electrodes are connected with an electrolytic apparatus in such a way, that as soon as the mercury rises and touches the left electrode, a current passes in the apparatus, the oxygen-producing side of which is connected with vessel *R*.

The electrolytic apparatus, containing a solution of 25% sulfuric acid, consists of a calibrated gas burette *A* and a vessel *B*. The latter is provided with a stopcock on top and with a side tube to the container *R*. Gas burette *A* and vessel *B* are connected with one another by a glass tube stoppered by a tight plug of fibrous asbestos soaked in the hydrolysis solution. No measurable volume of the solution can pass through this plug, whereas it is no obstacle for the electric current. The gas burette *A* and the vessel *B* each are provided with platinum electrodes. The electrode in *A* is connected with the negative side and that in *B*, via the electrodes in the capillary tube, with the positive side of an accumulator battery. When the current is passing, hydrogen is produced in *A* and oxygen in *B*. The volume of the hydrogen gas formed in *A* can be read in this calibrated burette, using the container *D*, connected with a rubber tube with *A*, as compensation vessel. The amount of oxygen gas formed in *B* is half of that of the hydrogen gas. This oxygen gas flows through the connecting tube to the container *A*, restoring the oxygen that has been taken up by the respiratory system in the soil sample. It is evident that at the start of the experiment the burette *A* must be filled to the top, while *B* must contain oxygen gas from the top to below the side tube.

The course of the experiment now is apparent. The soil sample takes up oxygen, and hence the pressure in *R* drops. As soon as the mercury in the capillary tube reaches the left electrode, an electric current passes and oxygen is formed in *B*. The gas pressure is hereby again restored and the mercury pressed back until the current is cut off and the liberation of oxygen stops. Cycles of consumption and restoration of oxygen in *B* and *R* follow one another at short intervals. The hydrogen formed in *A*, can be read at any time during the experiment. The oxygen consumption of the soil sample equals half the volume of hydrogen released. The carbon dioxide formed is estimated by titration of the alkali. The advantage of this apparatus is that any fluctuations in temperature and barometric pressure have no influence on the readings, provided that all parts of the apparatus are kept under the same conditions of temperature and pressure.

It must, however, not be forgotten that, besides respiration, other processes can take place in the soil. For instance the processes in the biological cycle of nitrogen compounds, such as nitrification, denitrification, ammonification and nitrogen fixation may occur. In all these cases exchanges in the gas sphere of the reaction vessel *R* will not reflect purely respiratory processes, and a thorough examination of the changes in the nitrogen compounds of the soil will be necessary.

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